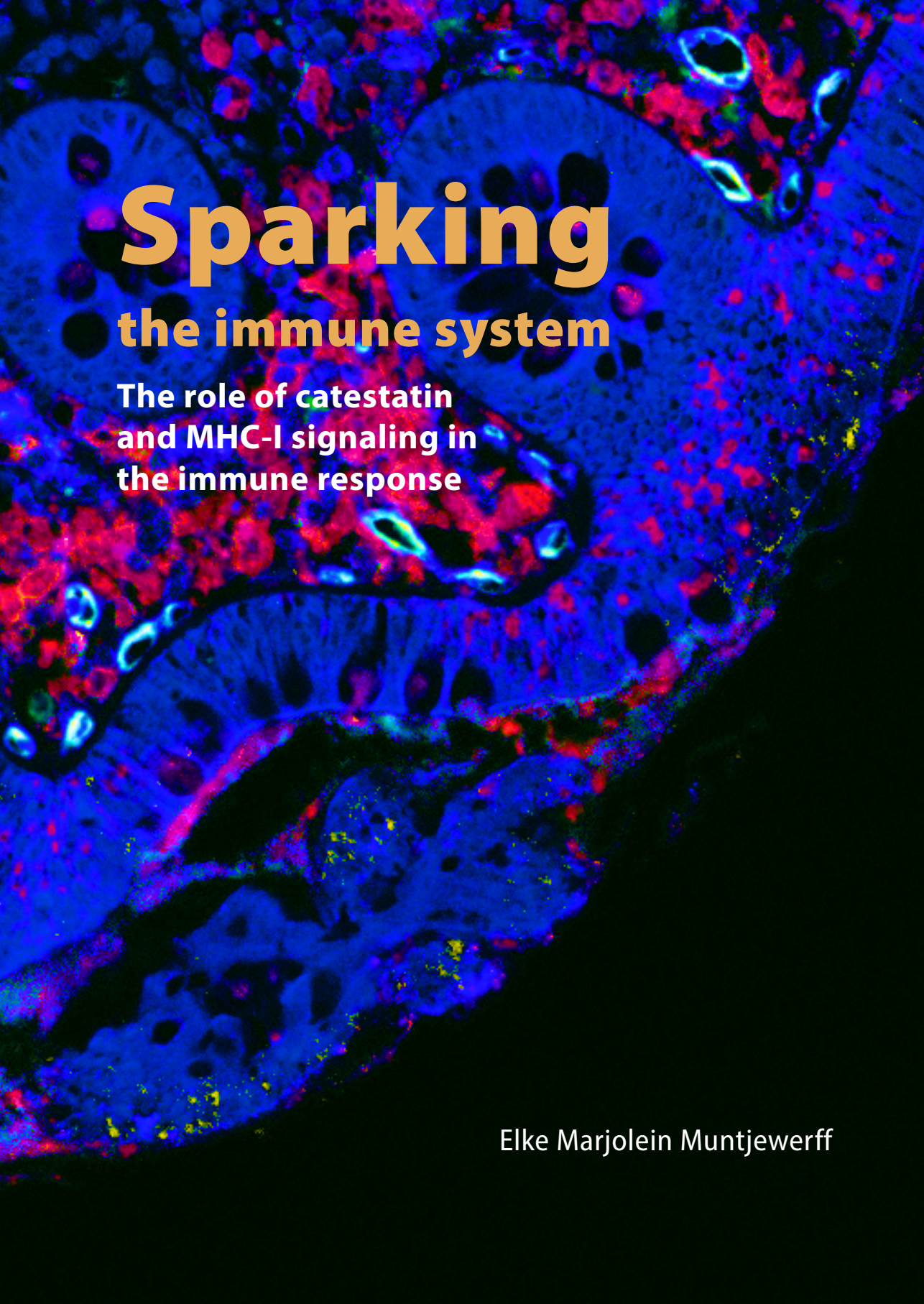

Sparking
the immune system

The role of catestatin and MHC-I signaling in the immune response

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The role of catestatin and MHC-I signaling in the immune response

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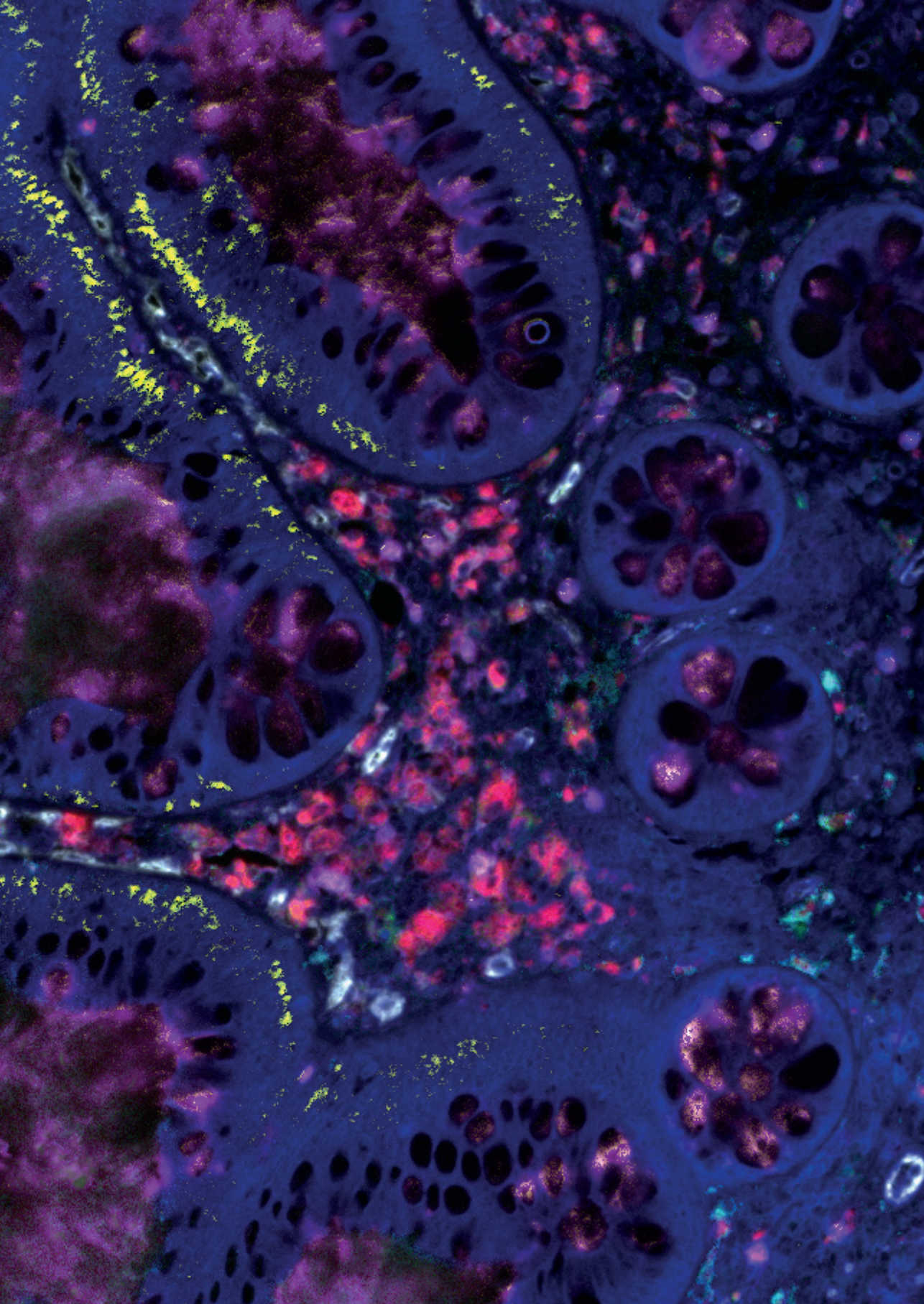

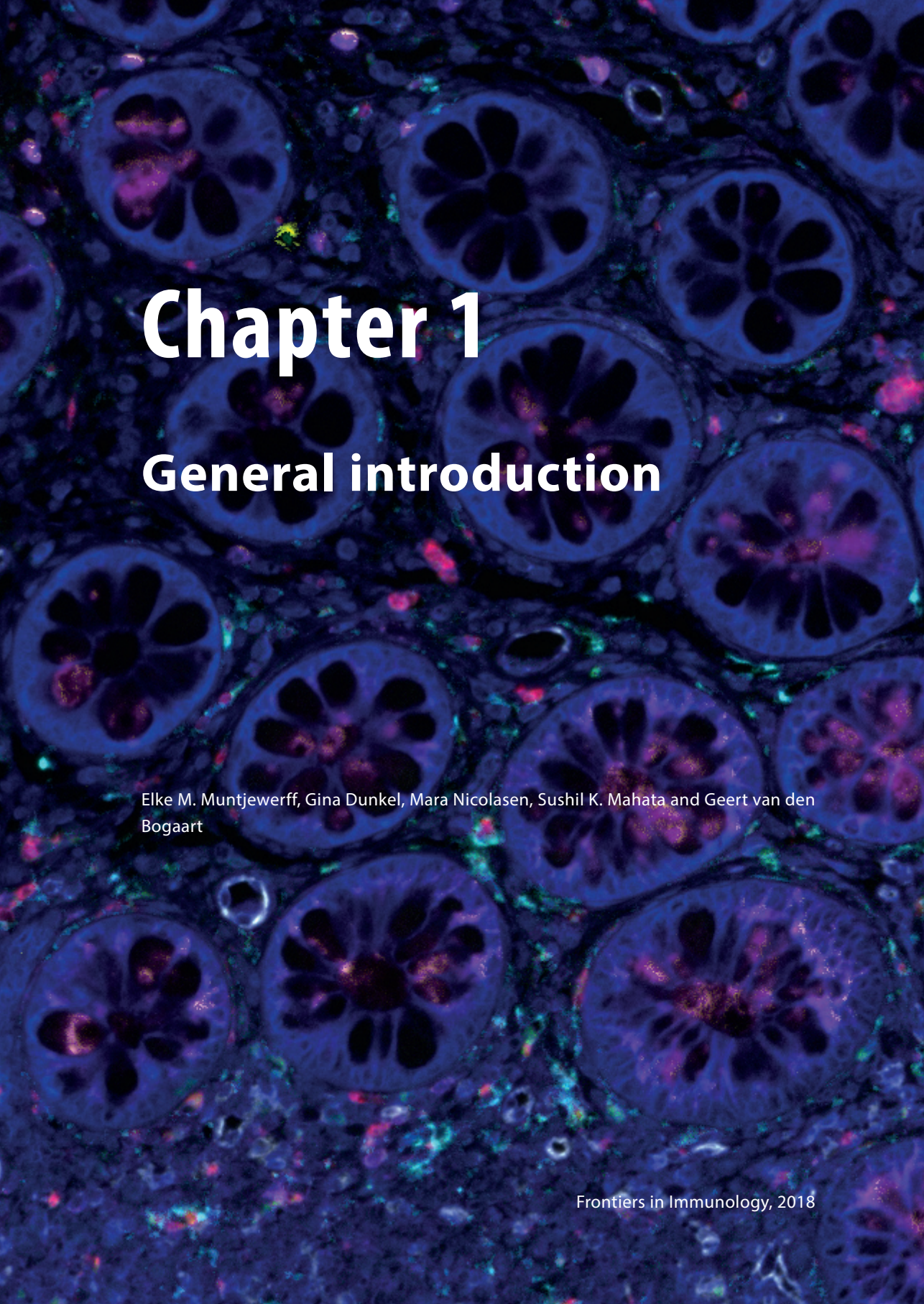

Chapter 1

General introduction

Elke M. Muntjewerff, Gina Dunkel, Mara Nicolasen, Sushil K. Mahata and Geert van den Bogaart

Frontiers in Immunology, 2018

Inflammation-based diseases, such as chronic inflammation (e.g., inflammatory bowel disease (IBD) and Type 2 diabetes Mellitus (T2DM)), auto-immune diseases (rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)), hypertension and severe cancer (myeloma, neuroendocrine tumors, lung and breast cancer) (1–3) are major health problems. For instance, two million people in the United States suffer from IBD, which includes patients with Crohn's disease (CD) and ulcerative colitis (UC) (4). Moreover, 415 million adults were globally affected by T2DM in 2015 and this caused 5.0 million deaths (5). For RA the current mortality rate is 2.2 million (6) and for SLE comorbidities including infection and cardiac malfunction account for 29% of all deaths (5,7). The second leading cause of death worldwide is cancer and 1 in 6 people die of cancer, accounting for 8.8 million deaths in 2015 (5). The prevalence of all these diseases is increasing and, in many cases, sufficient therapies are not available. Recently, an interest in utilizing the body's own molecules to treat these diseases arose. An interesting candidate for this is the pro-hormone chromogranin A (CgA), which contributes to a balanced immune response (8,9). CgA is proteolytically cleaved, both intracellularly as well as extracellularly after its release, and this gives rise to several bioactive peptides that can be released locally by endocrine cells in for example the pancreas or the gut (10,11). Moreover, CgA has been detected in other secretory vesicles of endocrine, neuroendocrine and neuronal tissues (12–15) as well as in keratinocytes (16), myocardial cells (17–19), endothelial cells (20,21), and macrophages (20). The bioactive peptides exert a broad spectrum of regulatory functions among the metabolic, endocrine, cardiovascular and immune systems (22). It is becoming increasingly clear that one of these cleavage products, the bioactive peptide catestatin (CST: $hCgA_{352-372}$) (23,24), is particularly of interest, since it suppresses tissue inflammation and affects the immune system. Indeed, the concept is emerging that CST plays an immunomodulatory role in the regulation of macrophages, metabolism, hypertension, insulin resistance and in gut homeostasis(8,9). Therefore, we investigated the new concept of modulating innate immunity by targeting CST, by examining the effect of CST on gut homeostasis and infiltrating immune cells and the role of CST in inflammatory bowel disease (IBD).

Gut homeostasis and chronic inflammation in the gut

The protection against pathogens, viruses and bacteria, already starts on the outside of our body. Invaders are stopped by several physical barriers including the skin, mucus and environment with a low pH, such as the stomach. The gastro-intestinal tract itself also contains beneficial bacteria that help to take up food and nutrients and complement the mucus layer(25). However, the balance between these 'good' and 'bad' bacteria in the gut is very important since an increase in for example, the negative bacteria *Bacteroidetes*, can disturb the protective mucus layer because *Bacteroidetes* metabolize the mucin glycans (26)(Fig. 1). Normally, the mucus layer prevents

the bacteria to reach the intestinal epithelial lining of the villi, which separates the lumen with bacteria from the underlying gut tissue (27). There are several cell types present in these intestinal villi such as goblet cells, which produce the mucus, and enteroendocrine cells (EECs). EECs are cells in the intestine helping the epithelium to regulate nutrient absorption, secretion and motility by producing several pro-hormones, including CgA (28,29). Hormones, such as glucagon-like peptide 1 and ghrelin, produced by EECs can access the central nervous system to regulate digestive processes, immune regulation, immune cell migration and gut motility (27,30). A dysregulation in these hormones contributes to intestinal inflammation (31,32), such as found in IBD.

IBD is a chronic inflammatory disorder with an incompletely understood pathogenesis, that is potentially linked to the gastrointestinal endocrine system (GES)(27). The two main forms of IBD are Ulcerative colitis (UC) and Crohn's disease (CD). UC is characterized by inflammation of the mucosa in the rectum and colon, whereas CD involves inflammation in all layers of the intestinal wall. In contrast to UC, CD is mainly found in the ileum, but can affect the whole gastrointestinal tract (27). In the colon of IBD patients, the epithelial layer is more permeable and leakier resulting in more exposure to pathogens which can lead to inflammation of the gut (Fig. 1). Monocytes, granulocytes and T cells are attracted from the blood to the inflamed area, followed by infiltration in the intestinal tissue.

Tissue macrophages such as liver Kupffer cells, spleen red-pulp and large peritoneal macrophages develop during embryogenesis, where they originate from precursors in the yolk sac, fetal liver and bone marrow (33). These embryonically-derived macrophages become tissue-resident macrophages that can propagate via self-renewal (33,34). Later in life, the hematopoietic stem cells in the bone marrow give rise to $LY6C^+$ monocytes in mice or $CD14^+CD16^+$ monocytes in human, which can subsequently be recruited from the blood into the tissues, such as sites of infection or tumors, to either promote further inflammation or tissue repair (34–36). Upon arrival, these monocytes can either remain monocytes, exerting for example antigen-presenting roles (37), and/or differentiate into macrophages upon external signals, PRR signaling, growth factors, and cytokines present in the tissue (38–40)these monocytes can differentiate into pro- or anti-inflammatory monocyte-derived macrophages, depending on the PRR signaling, growth factors and cytokines present in the tissue. Both monocytes and macrophages, (tissue-resident or monocyte-derived) can release inflammatory cytokines, including interleukin-1β (IL-1β), IL-6, IL-8, Tumor necrosis factor-alpha (TNF-α) as well as chemokines, such as CCL2 and CCL3 (38–40), which normally contribute to the elimination of pathogens. However, in the case of IBD the intestinal inflammation is not completely resolved, which eventually results in chronic inflammation.

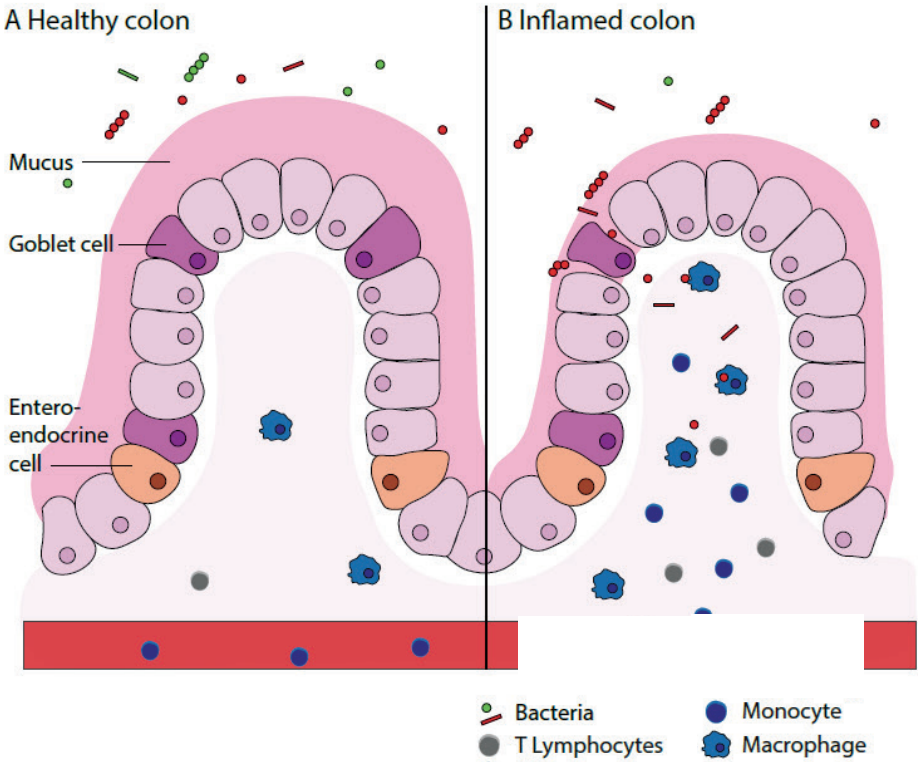

Fig. 1 Healthy vs inflamed colon. (**A**) Healthy colon: good (green) and bad (red) bacteria, shielded from intestinal cells by the mucus layer (pink), which is produced by goblet cells (purple), and includes enteroendocrine cells that produce hormones such as chromogranin A. Tissue resident macrophages (blue) and T cells (grey) (**B**) Inflamed colon: the bacteria composition has changed and the mucus layer is thinner. As a result, the bacteria can now infiltrate into the intestinal tissue. To eliminate the bacteria monocytes, monocyte-derived macrophages and T cells are attracted to the affected area.

So far, no cure has been found for IBD and regulation of gut homeostasis remains partly unknow. Since IBD is characterized by chronic inflammation and a dysregulated intestinal immune system (27), it is possible that the immunomodulatory pro-hormone CgA and its bioactive cleavage product CST are involved in the pathogenesis of IBD.

Chromogranin and its cleavage product Catestatin

The plasma (nM) and high local (μM) CgA or CST levels seem to change in health and disease (8). The CgA plasma levels range from 0.5 to 1 nM (22), whereas the physiological blood levels of CST range from 0.03 to 1.5 nM in healthy subjects (22,41). To understand the role of CgA and CST in colon homeostasis we will first take a more detailed look into the location and production of CgA and its cleavage products resulting in detectable plasma levels.

Cleavage products of the pro-hormone Chromogranin A (CgA)

The human CgA gene is located on chromosome 14 (42,43) and codes for a 439 amino acids long protein (44). As member of the granin family, CgA is characterized by an acidic pI, heat stability, and 8-10 pairs of dibasic cleavage sites (45). Moreover, this 49 kDa protein has the capacity to form aggregates and the ability to bind calcium (Ca^{2+}) with a high capacity, but low affinity (46). CgA was first identified as an acidic protein co-stored and co-released with ATP and catecholamines in chromaffin granules of neuroendocrine cells in the adrenal medulla (47,48). CgA facilitates the storage in these granules of catecholamines and ATP at hyperosmotic concentrations in a non-diffusible form (47–51). Thereby CgA contributes to the biogenesis of secretory granules packed with condensed proteins, mostly (pro) hormones (52,53) via recruitment of proteins involved in the formation and trafficking of vesicles, such as cytoskeleton-, GTP-, and Ca^{2+}-binding proteins (54). The secretory granules route toward the cell periphery, where they mature and undergo calcium-controlled exocytosis (55–57). Upon an increase in Ca^{2+} concentration, CgA is co-released simultaneously with the stored hormones of the secretory granules via exocytosis (55–57). CgA is not only present in chromaffin cells, but has been detected in other secretory vesicles of endocrine, neuroendocrine and neuronal tissues (12–15) as well as in keratinocytes (16), myocardial cells (17–19), endothelial cells (20,21), and macrophages (20). Interestingly, CgA is also present in cells of the pancreatic islet, secretory granules of glucagon containing α-cells and insulin producing β-cells, and may thereby modulate glucose metabolism (15,58–62). This makes CgA particularly interesting in the context of metabolic diseases such as diabetes. Patients suffering from carcinoids or other neuroendocrine tumors (55,63–67), heart failure, renal failure, hypertension, RA, and IBD (32,68–73) display increased levels of circulating CgA, implicating an important role of CgA to influence human health and disease (3). These functions indicate that the role of CgA is more general as a pro-hormone and is not only restricted to the storage of catecholamines.

Cleavage products of CgA

CgA can be proteolytically processed in various tissues and thereby serves as a precursor for several biological active peptides (Fig. 2). The cleavage of CgA at its dibasic sites is performed by intra-granular and extra-cellular proteases such as prohormone convertases 1 (PC1) (74), PC2 (74), furin (74), cysteine protease cathepsin L (CTSL) (75), the serine proteases plasmin (76,77) and thrombin (78), as well as by kallikrein (79). Depending on the cleavage sites, post-translational modifications (glycosylation and phosphorylation) and proteolytic processing, CgA can result in the following six biological active peptides (22,80). The first peptide identified was pancreastatin (PST) ($hCgA_{250\text{-}301}$), which has an opposing effect to insulin (62,81,82). WE-14 ($hCgA_{324\text{-}337}$) was identified in midgut carcinoid tumors and acts

as an antigen for the highly diabetogenic $CD4^+$ T cell clones (83–85). Chromofungin ($hCgA_{47-66}$) has antimicrobial effects as well as effects on innate immune regulation (86,87). Vasostatin ($hCgA_{1-76}$) has a vasodilative and anti-angiogenic as well as antiadrenergic functions (88–90). Serpinin ($hCgA_{402-439}$) regulates granule biogenesis (91) and acts as a myocardial ß agonist (92). Finally, the pleotropic peptide CST ($hCgA_{352-372}$) has mainly anti-inflammatory effects (11,93) and is the central focus of this thesis. Cleavage of CST results in Cateslytin ($hCgA_{344-358}$), which is the active domain of CST (94,95). CgA is unique as several of its peptides exhibit opposing counter-regulatory effects for fine-tuning and maintaining metabolic homeostasis. As for cardiac function, this is regulated in rodents by the pro-adrenergic peptide serpinin (92) and both antiadrenergic peptides vasostatin and CST (96,97). Likewise, angiogenesis is controlled by the antiangiogenic peptide vasostatin (98,99) and the proangiogenic peptide CST (98,100). Similarly, glucose homeostasis is maintained by the anti-insulin peptide pancreastatin (62,81,82,101), and the pro-insulin peptide CST (102). Although CgA processing has been reported to occur intracellularly inside the hormone-storage vesicles and extracellularly after its release in the blood, no systematic studies have been conducted to determine whether several proteolytic enzymes act at the same time to liberate all of the CgA peptides or act at different sites at different times in a tissue-specific manner. In addition, no attempts have been made so far to assess whether CgA peptides are generated in equal molar amounts or generated in response to physiological demands in different tissues. However, it has been reported that circulating concentrations of CgA peptides are different. As for example, plasma vasostatin level varies from 0.3 to 0.4 nM and CST circulates at 0.03 to 1.5 nM concentrations (22), which might represent different degrees of processing or rate of clearance from the circulation.

The pleotropic peptide catestatin (CST)

At first CST was identified as a potent inhibitor of nicotine induced catecholamine release. As CST is secreted together with catecholamines, it can thereby function as an autocrine negative feedback-loop self-limiting further catecholamine production (23,103–105). Later, CST was found to play a role in the regulation of hypertension(97,106–108) and cardiac functions (11,109–114), as well as in promoting angiogenesis (100,107), decreasing obesity(115) and regulating innate immunity (11,16,20,28,102,116,117). In line with this, altered plasma levels of CST or its prohormone CgA have been observed in the context of various diseases. Plasma levels of CST are reduced in patients suffering from T2DM and hypertension (41,102,118), whereas elevated levels of the pro-hormone CgA have been detected in the plasma of patients with neuroendocrine tumors (55), hypertension(119,120) and various inflammatory diseases such as RA (7,121,122), SLE (7), IBD (31,32,73,123,124) as well as T1DM and T2DM (85,125–128). This suggests that the lower levels of CST are caused

by a dysregulation of proteolytic processing of CgA (118). The balance between other CgA cleaved products seems also important to counteract effects of CST. Since vasostatin has an anti-angiogenic effect (88–90), this might counteract the pro-angiogenic effect of CST (100,107). Moreover, CgA-knockout (CgA-KO) mice develop an obese phenotype (62) as well as severe hypertension which could be rescued by intra-peritoneal injections of CST (106). Since hypertension is linked to diabetes, heart diseases and psoriasis (129), this indicates that CST might be important in various severe diseases. These findings support the hypothesis that the impaired processing of CgA might lead to lower CST levels which contributes to disease development. They also warrant further studies to elucidate the effects and mechanisms of CgA and its bio-active peptide products.

CST contributes to maintenance of metabolic and immune homeostasis

CST regulates immune homeostasis

CST contributes to the defense against infections in several ways (116,117,130). An early study utilized 15 amino acids from the N-terminal end of bovine CST or cateslytin to demonstrate the antimicrobial activities (116). Thus, CST can directly act on invading microbes, as CST can penetrate the membrane of bacteria and fungi. At relatively high concentrations (>μM) it thereby directly can impair the growth of fungal pathogens (116). Moreover, CST can induce lysis of bacteria and helps to protect against infections following skin injuries in mice (16). Second, at least *in vitro*, CST can result in activation of neutrophils and mast cells which contribute to innate immune responses to infections (28,116,117,130,131). These effects may be restricted to locally high CST concentrations, whereas systemic anti-inflammatory effects of CST have been best described in autoimmune diseases (Fig. 3).

In Diet-induced obese (DIO) mice, the intra-peritoneal injection of CST inhibited the infiltration of monocytes in the liver and reduced CC-chemokine ligand 2 (CCL2)-induced chemotaxis of peritoneal macrophages (102). The molecular mechanisms by which CST affects monocyte and macrophage migration are still unclear. One possibility is that CST directly affects leukocyte migration. This is shown for monocytes, although in this case already low concentrations of CST (nM) promoted migration in an *in vitro* chemotaxis assay (132). The reasons underlying this discrepancy between *in vitro* and *in vivo* experiments is unclear, but could be due to CST affecting other chemokines (such as CCL-2) present in the *in vivo* situation. Moreover, CST is also pro-angiogenic (100,107) which might reduce its anti-inflammatory effect when present on its own. Another possibility is that CST affects the integrins that affect leukocyte extravasation. This possibility is supported by the finding that CST can reduce expression levels of the integrin ligands intracellular adhesion molecule 1 (ICAM-1)

and vascular CAM-1 (VCAM-1) in endothelial cells, which correlate with lymphocyte extravasation (20). Finally, CST might reduce monocyte infiltration in inflammatory tissues by lowering the production of pro-inflammatory cytokines and chemokines by macrophages due to altered macrophage differentiation (28,102).

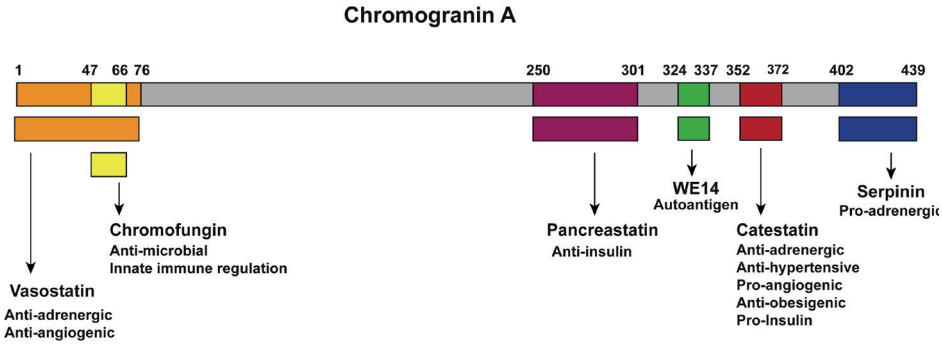

Fig. 2 Chromogranin A and the biological peptides. Cleavage of CgA gives rise to six known biological active peptides: vasostatin (Orange; $hCgA_{1-76}$), which has anti-adrenergic and anti-angiogenic functions(88–90); Chromofungin (Yellow; $hCgA_{47-66}$) has antimicrobial effects as well as effects on innate immune regulation; pacreastatin (Purple; PST; $hCgA_{250-301}$), which has anti-insulin functions(62,81,82); WE-14 (green; $hCgA_{324-337}$), which acts as an autoantigen for the highly diabetogenic CD4+ T cell clones(83–85); CST (Red; $hCgA_{352-372}$), which has pro-insulin, anti-obesigenic(115), pro-angiogenic(100,107), anti-adrenergic, anti-hypersensitive(97,106–108), cardiomodulatory(11,109–114) and anti-inflammatory functions(11,16,20,28,102,116,117); serpinin (blue; $hCgA_{402-439}$), which is pro-adrenergic and regulates granule biogenesis and acts as a myocardial ß agonist (91,92).

Considering the effects of CST on THP-1 cells (a human monocyte cell line), it seems that CST does not affect the overall differentiation of monocytes to macrophages. This was shown by consistent expression of the macrophage marker CD68 by THP-1 cells cultured with CST. However, CST steers the polarization of differentiation into less pro- and more anti-inflammatory phenotypes (20). CST treatment of THP-1 derived macrophages resulted in elevated levels of anti-inflammatory macrophage markers (mannose receptor C-type 1, MRC1) and reduced levels of pro-inflammatory macrophage markers (macrophage receptor with collagenous domain, MARCO) (20). Moreover, a reduction of pro-inflammatory gene expression (*TNF-α, F4/80, Itgam, Itgax, Ifng, Nos2* and *Ccl2*) was detected in isolated Kupffer cells and monocyte derived macrophages of DIO mice after intra-peritoneal injections with CST, whereas the levels of anti-inflammatory genes were increased (*IL-10, Mgl1, IL-4*, *Arg1* and *Mrc1*) (102). These results could be confirmed in isolated macrophages treated *in vitro* with CST (102). In the DIO mouse model, intra-peritoneal injections with CST also reduced plasma levels of pro-inflammatory cytokines and chemokines (TNF-α, INF-γ and CCL2) (102). Taken together, these findings indicate that CST shifts macrophage

differentiation from a pro- to a more anti-inflammatory phenotype. Since adipose tissue macrophages (ATMs) have antigen-presenting capacities this shift could influence the adaptive immune response (133). Interestingly, CD8 deficient mice show a decrease in macrophage infiltration and adipose tissue (134), suggesting that $CD8^+$ T cell infiltration precedes macrophage accumulation in inflammation. So, CST reduces inflammation, macrophage infiltration and might even influence the adaptive immune response by affecting T cell infiltration, but that would need to be validated in future experiments.

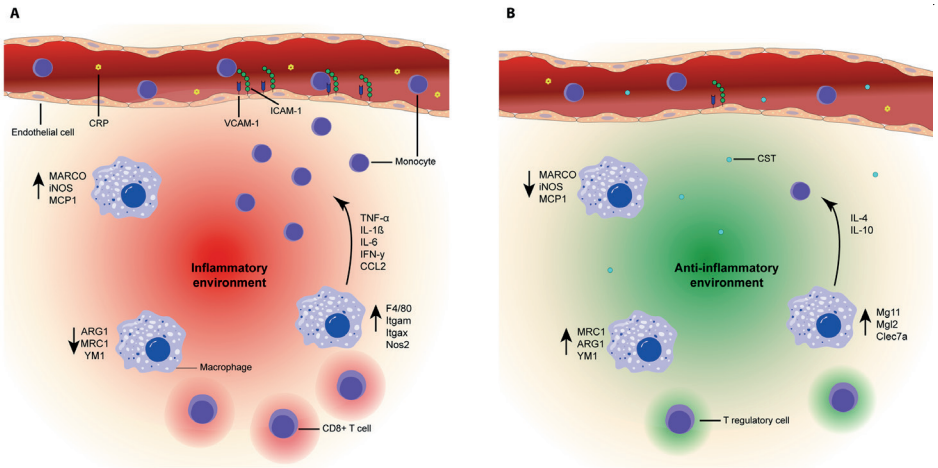

Fig. 3 Model of the suppressive effects of CST on inflammation. (**A**) Inflammatory state. High plasma levels of CRP (yellow) are present in an inflammatory state. Due to the presence of chemokines (CCL-2) and upregulation of integrin ligands (ICAM-1 (green) and VCAM-1 (blue)) on the endothelial cells (orange), increased monocyte (purple) infiltration is present at the inflammation site. An inflammatory environment (red) is created by the upregulation of pro-inflammatory markers (F4/80, Itgam, Itgax, NOS2, MARCO, iNOS, MCP1) in macrophages (blue). The production of inflammatory cytokines is also increased (TNF-a, IL-1β, IL-6, IFN-γ) and infiltration of $CD8^+$ T cells occurs. (**B**) CST treated state. Treatment with CST (light blue) results in lower levels of circulating CRP(28,135) and reduced expression of integrin ligands(20). Monocyte infiltration is reduced(20,28,102,136) and macrophages are polarized towards an anti-inflammatory phenotype, characterized by upregulation of anti-inflammatory markers (MRC1, ARG1, YM1, Mg11, Mgl2, Clec7a(20,102,136)) and increased production of IL-10 and IL-4(102,136). Moreover, expression of pro-inflammatory genes and cytokines are reduced(20,28,102,136). There will be no infiltration of $CD8^+$ T cells and Tregs might be present. Together, this contributes to an anti-inflammatory environment (green) and improved tissue architecture(20,28,136).

CST in intestinal inflammation

CST seems to have a specific anti-inflammatory effect in chronic gut inflammation as shown by several studies in IBD mouse models. In a colitis mouse model, intra-rectal injections with CST resulted in decreased serum levels of the acute phase reactant C-reactive protein (CRP) (28,135) and suppressed activity of myeloperoxidase (MPO),

which is a marker for granulocyte infiltration (28). As a result of these injections, the tissue architecture of the colon improved (28,136). Moreover, in atherosclerotic mice (apolipoprotein E-deficient mice), intraperitoneal injection followed by continuous subcutaneous CST infusion significantly retarded atherosclerotic lesions by 40% in the entire surface area of the aorta (20). In both colitis and atherosclerosis models, the prevalence of macrophages and monocytes in inflamed tissues was reduced following administration of CST (20,28,136), thereby supporting the anti-inflammatory effects of CST. Additionally, the gene expression levels of the pro-inflammatory macrophage markers inducible nitric oxygen synthase (*iNOS*) and monocyte chemoattractant protein 1 (*Mcp1*) were reduced upon intra-rectal injection of CST in a reactivated colitis mouse model, as well as *in vitro* in LPS stimulated peritoneal and colon macrophages (28,136). In both, the mouse model and *in vitro*, CST treatment resulted in decreased levels of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) (28,136). In the reactivated colitis mice model, CST promoted expression of several anti-inflammatory genes (*IL-10*, *Arg1* and *Ym1*) of the macrophages in the colon (136).

The pro-hormone chromogranin A (CgA) is produced by the endocrine cells of the GES that line the epithelium of the gastrointestinal tract (137). CgA is used as endocrine cell and serum marker in cancers(63), and might have a role in inflammatory and metabolic diseases such as diabetes (126,138) and IBD (32,73,123,124). CST mainly has anti-inflammatory properties, as exemplified by its ability to reduce inflammation in mouse models of UC (28,136,139) and other inflammatory diseases (8). Despite the counteracting activities of CgA and CST, blood plasma levels of both CgA (32,73,123,124) and CST (140) are increased in IBD patients. This raises the question how CgA and CST affect IBD pathology.

CST effects on metabolism

In addition to its anti-inflammatory effects, CST also affects metabolism. Opposite to insulin, CST inhibits lipogenesis and increases lipolysis in adipose tissue by inhibition of the α2-adrenergic receptor and by enhancing leptin signalling (115). Simultaneously, it stimulates fatty acid uptake and breakdown in the liver, as reflected by increased expression of the genes involved in fatty acid oxidation upon intra-peritoneal CST injections in mice (115). In line with this, CST injections in CgA-KO mice resulted in decreased triglyceride levels in the plasma and reduced fat depot sizes by ~ 25% (115). These findings indicate that CST promotes lipid flux from adipose tissue to the liver for beta oxidation, which might explain the frequently observed weight gain in patients with inflammatory diseases, as these patients have lower plasma levels of CST (41,102,118).

Besides the effect on lipid metabolism, intra-peritoneal administration of CST improved glucose and insulin tolerance in Diet-induced obese (DIO) mice and insulin-resistant systemic CST-KO mice, that express a truncated version of CgA (102). This could be due to CST inhibiting gluconeogenesis in the liver, thereby lowering the production and release of glucose in the blood (102). This effect of CST could be mediated by the modulation of Kupffer-cells and monocyte-derived macrophages, since the effects of their cytokines are linked to glucose and insulin metabolism (102,141) and for instance neutralization of TNF-α improves insulin sensitivity (142). Thus, CST can promote lipid and glucose metabolism, and thereby might help to prevent obesity and maintain homeostasis of metabolic functions (10,102,115). Although CST immunoreactivity has been detected in carcinoid tumours of the appendix, bronchus, stomach, small bowel and large bowel (143), its effects on cancer metabolism is yet to be investigated. However, insulin has been reported to promote cancer metabolism by upregulating pyruvate kinase M2 isoform (PKM2) expression and decreasing its activity, eventuating in amplification of cancer-metabolism-specific parameters like glucose uptake, lactate production, glycolytic pooling and macromolecular synthesis (144). In addition, several reports reveal increased cancer risk under hyperinsulinemic condition (145,146). Since CST decreases insulin level in hyperinsulinemic as well as insulin-resistant DIO and CST-KO mice (102), we expect that CST would decrease tumour growth by decreasing expression of PKM2 and increasing its activity, which requires experimental validation. Interestingly, PST, another cleavage product of CgA counteracts the metabolic and insulin sensitizing effects of CST (102). These anti-insulin actions of PST are likely important in maintaining homeostasis in glucose metabolism (10,62,101). The exact regulation of the proteolytic generation of CST and PST remains to be elucidated, but it could be coupled to metabolism via glycosylation because hyper-glycosylation of CgA is known to lead to reduced levels of CST (147). Thereby, the generation of CgA cleavage products might be regulated by sugar levels and this might play a role in progression of metabolic diseases, as for instance the increased blood glucose levels in T2DM might promote glycosylation of CgA.

Although it is increasingly clear that CST exerts anti-inflammatory effects on macrophages, the underlying mechanisms are still largely unknown. A key open question is to which receptor CST binds to exert its effect. This could either be a plasma membrane receptor as well as an intracellular target, since CST can penetrate the cell membrane of neutrophils (116,117). Another question is which signaling pathways are influenced by CST. Based on experiments with inhibitors in mast cells, CST treatment leads to cellular activation by mobilizing intracellular Ca^{2+} and inducing the production of pro-inflammatory cytokines/chemokines (GM-CSF, CCL2, CCL3 and CCL4) via a mechanism possibly involving G-proteins, phospholipase C and the

mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) (131). However, it is unknown whether these pathways are also responsible for the anti-inflammatory signaling in macrophages by CST.

Clinical implications of CST

Given its roles in metabolic regulation and immune homeostasis, CST has potential clinical applications as a diagnostic marker and even as a therapeutic target. For example, lower levels of CST have been reported in the blood of patients suffering from T2DM, suggesting that it might be a diagnostic marker for this disease (41,102,118). However, it might be more useful to study CST levels relative to other cleavage products of CgA, considering that some of these cleavage products counteract the activities of CST. For instance, PST exerts opposing effects on insulin sensitivity and glucose metabolism compared to CST (81), and increased levels of PST can contribute to T2DM (61). The observed lower levels of CST might well be caused by dysregulation of proteolytic processing of CgA (118), since this could result in a higher ratio of PST to CST. Indeed, an altered processing of CgA has been observed in the microenvironment of tumors. Here CgA cleavage products lead to proangiogenic activity, as cleavage of the N- and C-terminal regions of CgA can activate antiangiogenic (vasostatin) and proangiogenic sites (CST), respectively (1). Further supporting the notion that CgA and its cleavage products can be diagnostic markers for various diseases, is that elevated levels of CgA have been detected in the plasma of patients with neuroendocrine tumors(55), hypertension(119,120) and various inflammatory diseases such as RA (7,121,122), SLE (7), IBD (31,32,73,123,124) as well as T1DM and T2DM (85,125–128). However, not all assays used in the aforementioned studies allow to discern full-length from proteolytically processed CgA (148) and it would be very interesting to compare this to levels of unprocessed CgA and its cleavage products.

As discussed above, CST can decrease inflammation by reducing immune infiltration in inflamed tissues and altering macrophages differentiation into an anti-inflammatory phenotype (28,62,102,118,136,148). These effects are already observed at concentrations in the nM range, which corresponds to physiological concentration levels of circulating CST (22). By lowering the production of pro-inflammatory cytokines, CST may suppress inflammatory immune responses and/or might promote the dissolvement of inflammation. As a result, CST could prevent chronic states of inflammation and inhibit exaggerated inflammatory responses normally leading to tissue damage. Although CST exerts primarily anti-inflammatory effects, other cleavage products of CgA have opposing pro-inflammatory effects. Disbalances in the levels of circulating CgA-derived peptides might therefore contribute to various diseases including IBD (3).

Scope of this thesis

In this thesis, we investigated the relatively new concept of modulating innate immunity by targeting CST, which may find applications in treatment of various inflammatory based diseases and cancer (1–3). Here, we examined the effect of CST on gut homeostasis, infiltrating immune cells and the role of CST in inflammatory bowel disease (IBD).

In **chapter 2**, we examined the role of CgA and CST on intestinal barrier function and inflammation, by investigating the influence of CST and CgA on tight junctions and permeability, using ultrastructural studies and plasma FITC-dextran assays in knockout mice for CgA or CST. The observed leaky gut in CST-KO mice was associated with a higher ratio of Firmicutes to Bacteroidetes, a dysbiotic pattern commonly encountered in IBD. Supplementation of CST-KO mice with recombinant CST reversed this leakiness and key readouts. Supplementation of CgA-KO mice with either CST alone, or with the pro-inflammatory proteolytic CgA fragment pancreastatin (PST: $CgA_{250\text{-}301}$) showed that gut permeability is regulated by the antagonistic roles of these two peptide hormones: PST increases and CST reduces leakiness. We conclude that the enteroendocrine cell-derived hormone, CgA regulates post-prandial gut permeability. CST is both necessary and sufficient to reduce the leakiness. CST acts primarily *via* antagonizing the effects of PST.

Moreover, in **chapter 3** we took a closer look at the colon histology of CST-KO mice and patients with IBD to observe the beneficial effects of CST in an inflammatory environment. Here we show that bone-marrow derived immune cells regulate the colonic mucus layer by production of CST, a bioactive peptide derived from the pro-hormone CgA. Since IBD patients have elevated stool CST this might contribute to the reduced mucus layer in inflamed colon regions. Additionally, in **chapter 4,** we report that CST blocks leukocyte migration towards inflammatory chemokines. By *in vitro* and *in vivo* migration assays, we show that although CST itself is weakly chemotactic, it blocks migration of monocytes and granulocytes to inflammatory attracting factor CC-chemokine ligand 2 (CCL2) and macrophage inflammatory protein 2 (MIP-2). Moreover, it directs CX_3CR1^+ macrophages away from pancreatic islets. These findings support the emerging notion that CST is a key anti-inflammatory modulator.

In the second part of this thesis, we focus more on the information exchange between antigen-presenting cells and cytolytic T cells. We will introduce this topic in **chapter 5 & 6,** followed by the actual research on the immunological synapse in **chapter 7**. In **chapter 5** we briefly discuss the role of macrophage antigen cross-presentation to the activation of cytolytic T cells for immune defenses against tumors, viruses and

intracellular pathogens. Here we provide an overview of *in vitro* and *in vivo* evidence on cross-presentation by various types of tissue-resident and circulating macrophages via similar cellular pathways as DCs. Since macrophages can cross-present and thereby might aid in CTL responses, stimulating macrophages to cross-present might be a promising strategy for anti-tumor or anti-viral therapies. Hereafter, in **chapter 6**, we give an overview of mechanism as well as the function of MHC-I reverse signalling, which is involved in the defense regulation against malignancies and viral or bacterial infections. We discuss and compare this signalling in multiple cell types, ranging from immune cells such as macrophages, natural killer (NK) cells, T- and B-cells to non-immune cells like endothelial, smooth muscle cells and cancer cells. Followed by **chapter 7**, where we examined the effects of MHC-I on immunological synapse formation in dendritic cells. Here, we used electron microscopy to visualize the microtubule organizing center (MTOC) at the immunological synapse between the DC and T cell. Followed by bead assays coated with various T cell receptors showing that MHC-I drives IL-12 release via MTOC polarization.

Chapter 8 provides a brief summary of the main findings of this thesis as well as an extensive discussion about the possible working mechanism of CST in macrophages and neuroendocrine cells via autocrine/paracrine signaling. Moreover, we will discuss further directions in CST research and therapeutic applications.

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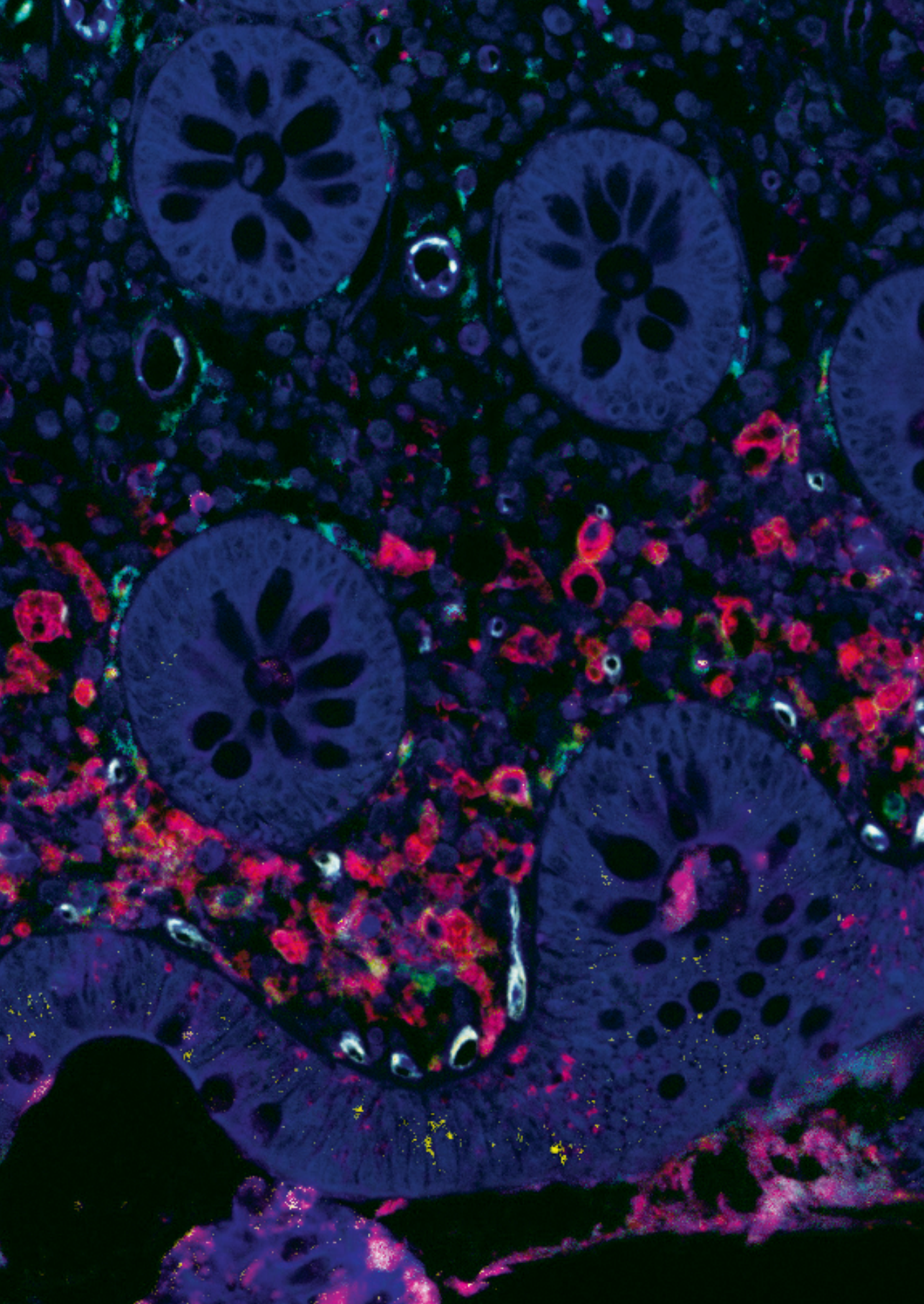

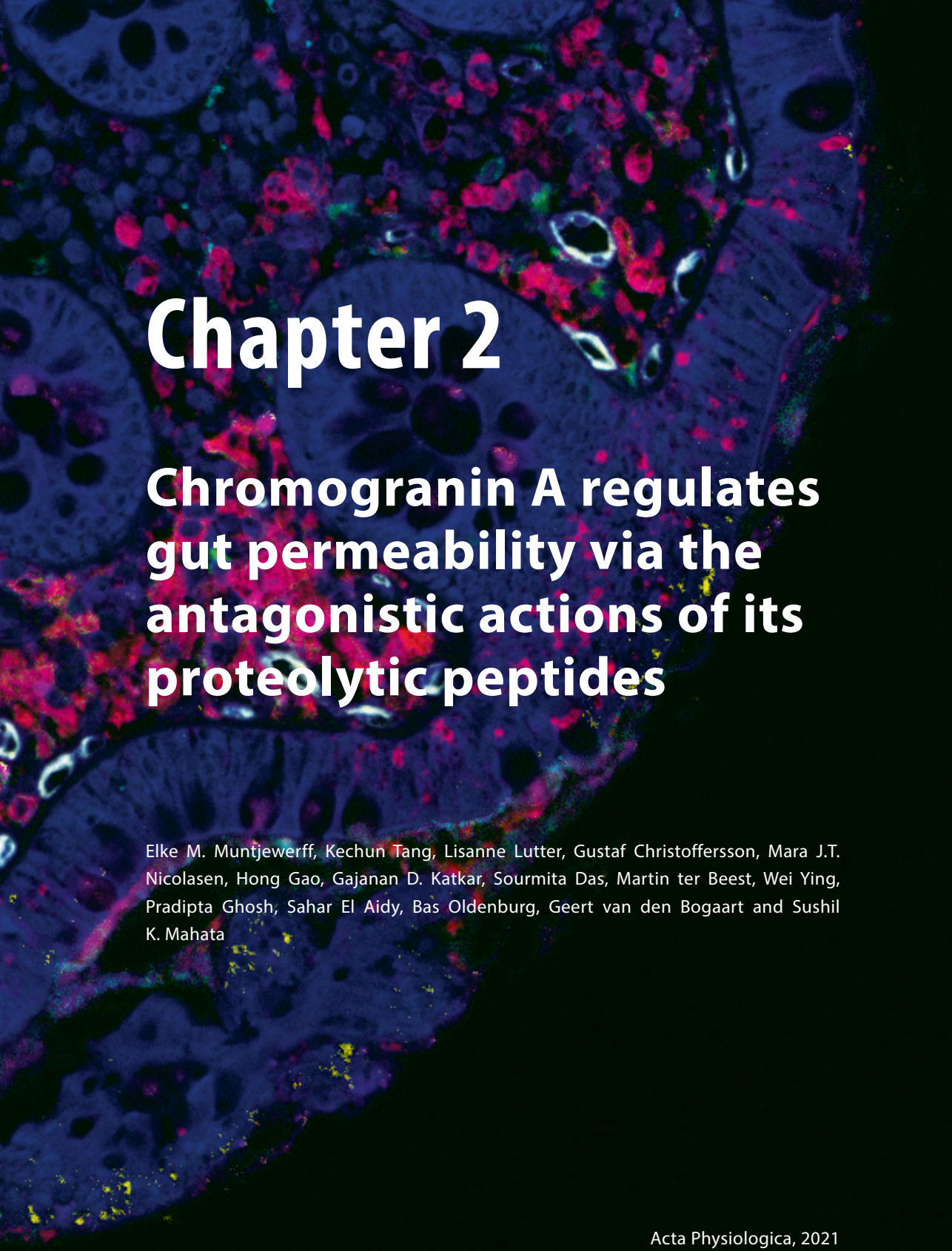

Chapter 2

Chromogranin A regulates gut permeability via the antagonistic actions of its proteolytic peptides

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Abstract

Aim: A 'leaky' gut barrier has been implicated in the initiation and progression of a multitude of diseases, e.g. inflammatory bowel disease (IBD), irritable bowel syndrome and celiac disease. Here we show how pro-hormone Chromogranin A (CgA), produced by the enteroendocrine cells, and Catestatin (CST: $hCgA_{352-372}$), the most abundant CgA-derived proteolytic peptide, affect the gut barrier.

Methods Colon tissues from region-specific CST-knockout (CST-KO) mice, CgA-knockout (CgA-KO) and WT mice were analyzed by immunohistochemistry, Western blot, ultrastructural and flowcytometry studies. FITC-dextran assays were used to measure intestinal barrier function. Mice were supplemented with CST or CgA fragment pancreastatin (PST: $CgA_{250-301}$). The microbial composition of cecum was determined. CgA and CST levels were measured in blood of IBD patients.

Results: Plasma levels of CST were elevated in IBD patients. CST-KO mice displayed (i) elongated tight, adherens junctions and desmosomes similar to IBD patients, (ii) elevated expression of Claudin 2, and (iii) gut inflammation. Plasma FITC-dextran measurements showed increased intestinal paracellular permeability in the CST-knockout mice. This correlated with a higher ratio of Firmicutes to Bacteroidetes, a dysbiotic pattern commonly encountered in various diseases. Supplementation of CST-knockout mice with recombinant CST restored paracellular permeability and reversed inflammation, whereas CgA-knockout mice supplementation with CST and/or PST in CgA-KO mice showed that intestinal paracellular permeability is regulated by the antagonistic roles of these two peptides: CST reduces and PST increases permeability.

Conclusion: The pro-hormone CgA regulates the intestinal paracellular permeability. CST is both necessary and sufficient to reduce permeability and primarily acts by antagonizing PST.

Introduction

The intestinal tract harbors 10^3-10^{11} bacteria/ml [1] that in aggregate can constitute >1 g of endotoxin [2]. Endotoxins stimulate immune cells in the lamina propria, which can trigger mucosal inflammation [3]. Such inflammation can result in luminal dysbiosis [4] and gut barrier defects [5-8]. The gut barrier is comprised of a surface mucus layer which separates intact bacteria and large particulates from the mucosa [9] and the colonic epithelium which provides a physical and immunological barrier separating the body from the luminal contents (e.g. dietary antigens, a diverse intestinal microbiome, and pathogens) [10-12]. Mucosal homeostasis is maintained by the delicate and complex interactions between epithelial cells, immune cells, and luminal microbiota [10].

The gastrointestinal epithelium not only forms the body's largest interface with the external environment, but also establishes a selectively permeable epithelial barrier; it allows nutrient absorption and waste secretion while preventing the entry of luminal contents. The epithelial barrier is regulated by a series of intercellular junctions between polarized cells: an apical tight junction (TJ), which guards paracellular permeability, the subjacent adherens junction (AJ) and desmosomes; the AJs and desmosomes do not seal the paracellular space but provide essential adhesive and mechanical properties that contribute to paracellular barrier functions [11].

The permeability of the barrier is regulated by many factors such as the immune system. For example, tumor necrosis factor (TNF)-α increases the flux of larger molecules, such as proteins and other macromolecules, through the leak pathway, and interleukin (IL)-13 selectively enhances the flux of small molecules and ions via the pore pathway [5]. TNF-α plays crucial roles in the Caveolin-1-mediated internalization of Occludin (OCLN), which elevates gut permeability. Conversely, overexpression of OCLN alleviates the cytokine-induced increase in gut permeability [6]. Interferon (IFN)-γ increases gut permeability by reducing the expression of Tight junction protein ZO-1 (TJP1) and OCLN as well as by modulating actin-myosin cytoskeleton interactions with TJ proteins [7]. The simultaneous presence of TNF-α and IFN-γ deteriorates intestinal integrity by dissociating TJ proteins [8].

Here we show that the permeability of the gut barrier is regulated by the pro-hormone Chromogranin A (CgA). CgA is an acidic secretory proprotein [13] that is abundantly expressed in enteroendocrine cells (EECs) of the gut and serves as a common marker for the EECs of the gut [14]. Originally, CgA was identified as a cellular packaging factor [15-17]. However, the pro-hormone CgA is proteolytically cleaved, both intracellularly as well as extracellularly after its secretion, giving rise to seven bioactive peptides that exert a wide variety of regulatory functions among the metabolic [18-21], cardiovascular

[22-25] and immune systems [26-30]. Among these bioactive peptide hormones are catestatin (CST: $hCgA_{352-372}$) [31,32] and pancreastatin (PST: $hCgA_{250-301}$) [33]. Blood plasma levels of both intact CgA [34-36] and CST [37] are increased in patients with inflammatory bowel disease (IBD). We show that CST is abundantly generated in the gut. Unexpectedly, we found that CgA regulates the paracellular permeability of the gut via the antagonistic actions of its two peptides, PST and CST; while PST is a pro-inflammatory, pro-obesigenic and anti-insulin peptide [20] which loosens the barrier, CST acts as anti-inflammatory, antiobesigenic, anti-microbial, and proinsulin peptide [25,27,29] that appears to be necessary and sufficient to neutralize the actions of PST and tighten the barrier. Using knockout (KO) mice in conjunction with recombinant proteins, we show that CST exerts its actions *via* counteracting PST.

Results

CgA is extensively processed to CST

To study the role of CgA and its peptides in the regulation of the gut barrier, we used systemic CgA-KO (deletion of exon 1 and ~1.5 kbp proximal promoter, thereby completely inactivating the *Chga* allele) [22] and CST-KO mice (the 63 bp CST domain of CgA was deleted from Exon VII of the *Chga* gene) [38]. CST-KO mice show low grade inflammation in the heart, pancreas, and liver and have an elevated blood pressure, are obese and suffer from insulin resistance [27,38]. We analyzed the function and morphology of the gut barrier in CST-KO mice. We used transmission electron microscopy (TEM) to resolve the ultrastructure of EECs and to evaluate the subcellular compartment where hormones are stored and released, i.e., dense core vesicles (DCVs). While DCV numbers were reduced in both CgA-KO and CST-KO mice, DCV areas were smaller in CgA-KO mice but larger in CST-KO mice (Fig. 1A&B). WT colon showed a lower concentration of CgA (average 0.019 nmol/mg protein) than of CST (average 0.37 nmol/mg protein), indicating extensive processing of CgA to CST (Fig. 1C). As anticipated, in CST-KO mice the levels of CgA in the colon were similar to that in WT mice, but those of CST were undetectable (Fig. 1C). Consistent with enhanced processing of CgA, we found increased expression of the proteolytic enzymes Cathepsin L (*Ctsl*, which cleaves CST from CgA) and Plasminogen (*Plg*, the precursor of plasmin, which is involved in CgA processing) in CST-KO and CgA-KO, respectively (Fig. 1D) [39,40].

Because the processing of CgA to CST is compromised in several diseases [41-43], we determined plasma CgA and CST levels in patients with Crohn's disease and with ulcerative colitis, both with active disease and in remission (Fig. 1E&F). In line with previous findings [34-36], we found higher CgA levels in plasma of patients with active Crohn's disease but not in patients in remission. In contrast, we found higher plasma

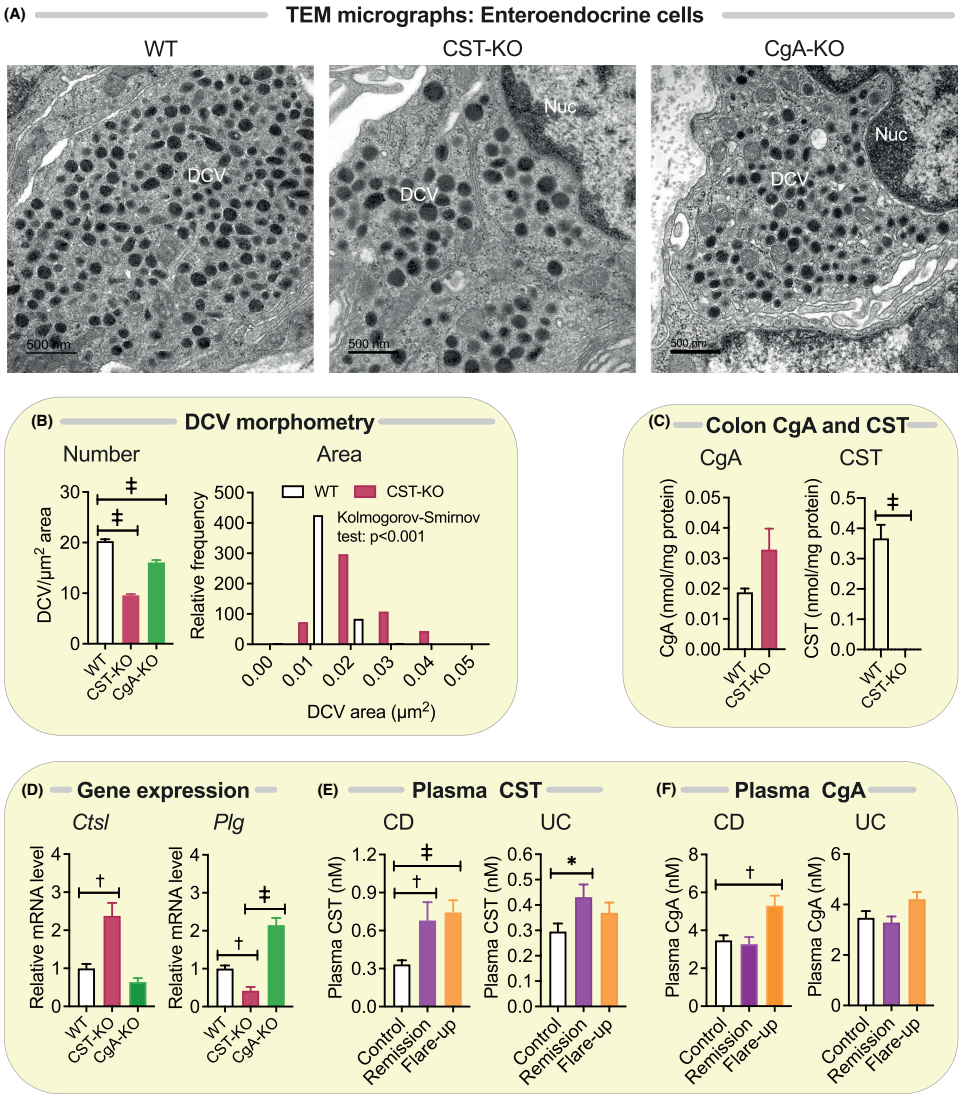

Fig. 1. CgA is efficiently processed to CST in the colon and CST levels correlate with Crohn's disease activity. (A) TEM micrographs (n=3) showing enteroendocrine cell DCV in colon of WT, CgA-KO and CST-KO mice, **(B)** fewer DCV in CgA-KO and CST-KO mice (1-way ANOVA) but enlarged DCV in CST-KO mice and smaller DCV in CgA-KO mice (>500 vesicles from 30 micrographs) (Kolmogorov-Smirnov test). DCV, dense core vesicles; Nuc, nucleus. **(C)** CgA protein and CST peptide levels in colon of WT and CST-KO mice. CgA levels were comparable between WT and CST-KO mice. CST level was markedly higher in WT mice and was undetectable in CST-KO mice (n=6; Welch's *t* test). **(D)** Expression of proteolytic genes (n=7). *Ctsl* and *Plg* are differentially expressed in colon of CgA-KO and CST-KO mice (n=8; 1-way ANOVA). **(E)** CgA and CST levels in EDTA-plasma of healthy donors (Control) and Crohn's disease patients (N=89) in remission or flare-up (2-way ANOVA). Ns, not significant; †, p<0.01; ‡, p<0.001.

CST levels in Crohn's disease regardless of disease activity, indicating that the processing of CgA to CST is more efficient in active disease. Patient characteristics are provided in Supplemental Table S1&S2.

CST-KO mice have a 'leaky' gut

Ultrastructural evaluation of the gut epithelium revealed that the TJs, AJs and desmosomes are significantly different in CST-KO mice compared to wild-type (WT): the TJs, AJs and desmosomes were elongated, as also observed in IBD patients (Fig. 2A, C-E, Fig. 3H-L). We determined whether this altered morphology was caused by an altered expression of proteins involved in these epithelial junctions. Gene expression studies showed a trend towards mRNA expression of multiple TJ-markers such as Occludin (*Ocln*), MARVEL domain-containing protein 2 (*Marveld2*, also called Tricellulin), Junctional adhesion molecule A (JAM-A; *F11r*) Tight junction protein ZO-1 (*Tjp1*) [44] and the desmosomal protein Desmoglein 2 (*Dsg2*), which is required for maintenance of intestinal barrier function [45]. We observed concomitant elevated mRNA expression of the genes coding for TJ components Claudin 1 and 2 (*Cldn1* and *Cldn2*), TJ protein ZO-2 and 3 (*Tjp2* and *Tjp3*), and AJ genes coding for alpha-E-Catenin (*Ctnna1*), Cadherin-associated protein (*Ctnnb1*), and Cadherin 1 (*Cdh1*) (Fig. 2F&G). Primer sequences are provided in Supplemental Table S3. For Occludin, ZO-1, alpha-E-Catenin, Cadherin 1, Claudin 1, we did not observe significantly altered protein levels. However, we did find expression at both the mRNA and protein levels of *Cldn2* (Fig. 2F, Fig. 3A&G), a specific claudin that is known to increase the paracellular flux of cations and water through the pore pathway [46]. In order to assess the functional consequence of the altered cellular junctions, we determined the flux through the leak pathway, which is responsible for the paracellular movement of larges molecules, including microbial proteins and other macromolecules. Compared to WT mice, we observed a more than 10-fold increase in leakage of plasma FITC-dextran (4 kDa) in the CST-KO mice (Fig. 2B). This confirms that the intestine in CST-KO mice has a strongly diminished paracellular barrier via the leak pathway, although the large magnitude of leakage might even suggest direct epithelial damage.

CST-KO mice and IBD patients show comparable ultrastructural changes in tight junctions, adherens junctions, and desmosomes.

Ultrastructural analyses of colonic mucosa revealed increased lengths of TJ, AJ, and desmosomes in IBD patients compared to healthy controls (Fig. 3H-L). Unlike CST-KO mice, IBD patients displayed a decrease in AJ diameter (Fig. 3H&K).

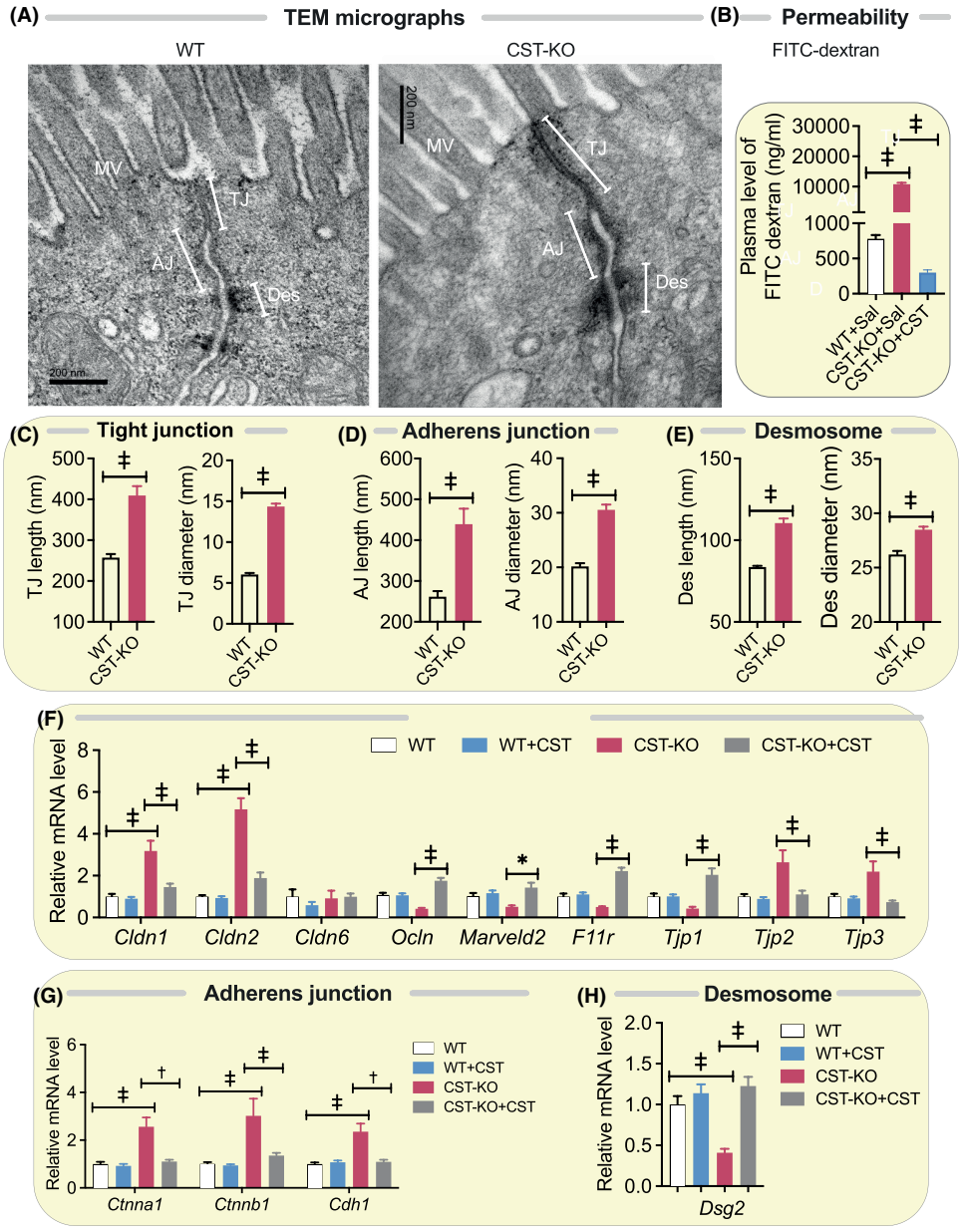

Fig. 2. Impaired epithelial paracellular barrier function in CST-KO mice. (A) TEM micrographs (n=3) showing tight junction (TJ), adherens junction (AJ), and desmosomes (Des). MV, microvillus. **(B)** Increased gut permeability as measured by FITC-dextran level in CST-KO compared to WT mice (n=8; Welch's *t* test). (**C-E**) Morphometrical analyses (n=20-54) showing increased length and diameter of TJ **(C)**, AJ **(D)** and Des **(E)** in CST-KO mice (Welch's *t* test). **(F-H)** Differential expression of genes in TJ (*Cldn1, Cldn2, Cldn6, Ocln, Marveld2, F11r, Tjp1, Tjp2, and Tjp3*), AJ (*Ctnna1*, *Ctnnb1*, and *Cdh1*) and Des (*Dsg2*) in colon of CST-KO mice compared to WT mice. Supplementation of CST-KO mice with CST reversed the expression of the above genes (n=8; 2-way ANOVA or Welch's *t* test). *, p<0.05; †, p<0.01; ‡, p<0.001.

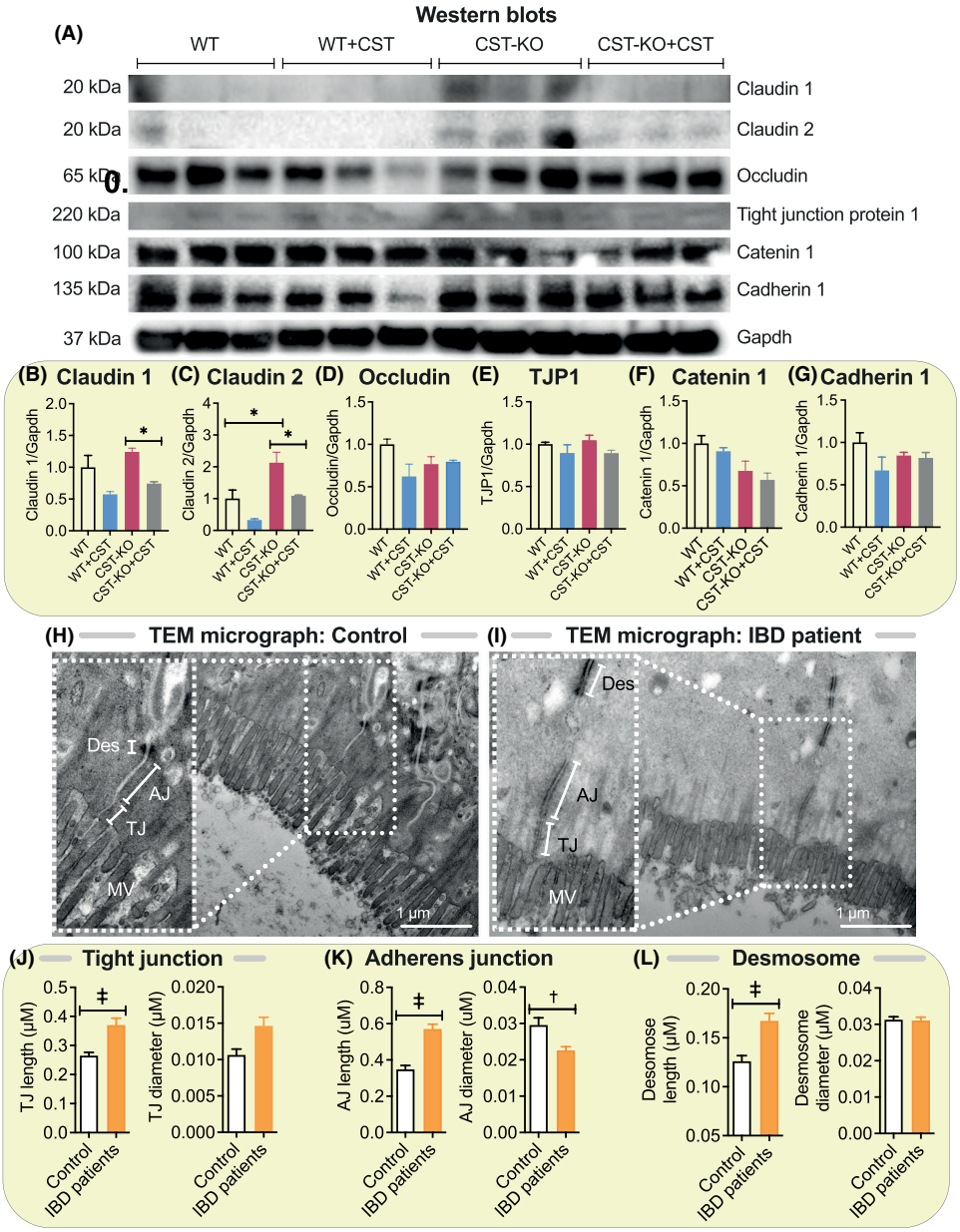

Fig. 3. Elevated levels of Claudin-2 in colon of CST-KO mice and elongated epithelial junctions in IBD patients. (A) Western blots and **(B)** densitometric analyses of Claudin-1 **(B)**, Claudin-2 **(C)**, Occludin **(D)**, Tjp1 **(E)**, Catenin 1 **(F)**, and Cadherin 1 (**G**). (**H&I**) TEM micrographs (n=3) showing tight junction (TJ), adherens junction (AJ), and desmosomes (Des) in healthy control and IBD patients. Microvillus (MV). (**J-L**) Morphometrical analyses (n=20-54) showing increased lengths and diameter of TJ (**J**), AJ (**K**) and Des (**L**) in IBD patients. *, p<0.05; †, p<0.01; ‡, p<0.001.

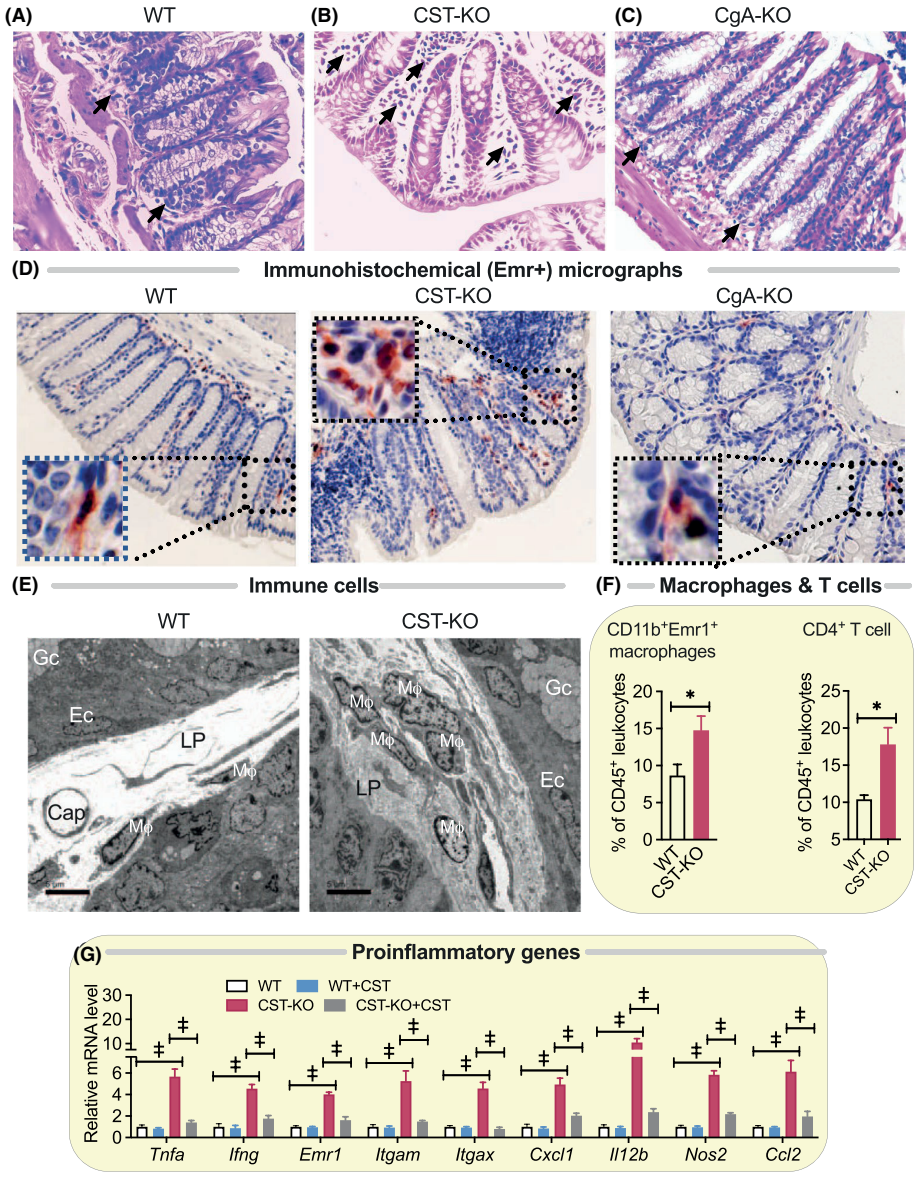

Fig. 4. Increased infiltration of immune cells in colon of CST-KO mice. (A-C) hematoxylin and eosin-stained histological micrographs of colon in WT **(A)**, CST-KO **(B)**, and CgA-KO **(C)** mice. Arrows indicate immune cells. **(D)** Immunohistological micrographs showing increased infiltration of Emr^+ cells in CST-KO mice. **(E)** TEM micrographs (n=3) showing increased number of immune cells in colon of CST-KO mice. Mϕ, macrophage; Cap, capillary; Ec, enterocytes; Gc, goblet cells; LP, lamina propria. **(F)** Flow cytometry study (n=5-10) shows increased number of macrophages ($CD45^+CD11b^+Emr1^+$) and T cells ($CD45^+CD4^+$) in colon of CST-KO mice (n=5-10; Welch's *t* test). **(G)** Increased expression of proinflammatory genes (*Tnfa, Ifng, Cxcl1, Ccl2, Emr1, Il12b, Nos2, Itgam*, and *Itgax*), which returned to WT levels after treatment with CST (n=8; 2-way ANOVA). (n=8; 2-way ANOVA). *, $p<0.05$; †, $p<0.01$; ‡, $p<0.001$.

The leaky gut in CST-KO mice is accompanied by mucosal inflammation and dysbiosis

Because inflammation regulates the epithelial cellular junctions [5-8] and an increased gut permeability is often associated with mucosal inflammation, we investigated the immune cell populations in the colons of CST-KO mice by histochemical analysis (Fig. 4A-C). Indeed, the colon of CST-KO mice displayed signs of mucosal inflammation, such as elevated immune cell infiltration (Fig. 4D&E), increased fibrosis (Fig. 5D&E), and increased apoptosis (Fig. 6A). In addition, there was increased abundance of macrophages ($CD45^+11b^+Emr1^+$) and helper $CD4^+$ T cells in the colon of CST-KO mice as confirmed by flow cytometry (Fig. 4F). Moreover, CST-KO mice showed increased expression of the following proinflammatory genes: TNF-α (*Tnfa*), IFN-γ (*Ifng*), Chemokine CXC motif ligand 1 (*Cxcl1* aka *Kc/Gro1*), Chemokine CC motif ligand 2 (*Ccl2* aka *Mcp1*), Egg-like module-containing mucin-like hormone receptor 1 (*Emr1* aka *F4/80*), Integrin alpha-M (*Itgam* aka *Cd11b*), and Integrin alpha-X (*Itgax* aka *Cd11c*) (Fig. 4G). Increased expression of *Tnfa, Ifng, Cxcl1*, and *Ccl2* genes correlated with increased protein levels (Fig. 5A). Upregulation of proinflammatory cytokines is in line with prior work demonstrating a positive correlation of IFN-γ [47] and TNF-α [44] with intestinal leakiness. The decreased expression of anti-inflammatory cytokines, such as interleukin 4 (*Il4*) and interleukin 10 (*Il10*), in CST-KO mice is also in congruence with the reported role of anti-inflammatory cytokines in gut permeability (Fig. 5C) [48]. The decreased *Il10* mRNA levels correlated with a decreased protein level of IL-10 (Fig. 5C). Furthermore, the expression of *S100a9* gene (forming Calprotectin in complex with S100a8) was increased in colon of CST-KO mice and Calprotectin protein levels were increased in plasma, colon and feces of CST-KO mice (Fig. 5B); increased Calprotectin, a protein secreted by activated neutrophils and macrophages, has been shown to be associated with increased intestinal permeability [49]. The increased inflammation in the gut of CST-KO mice is consistent with the known anti-inflammatory roles of CST [29,50] and with our previous observation of low-grade systemic inflammation in the CST-KO mice [27].

Because a leaky gut may either cause or be the consequence of altered luminal microbes [51], we investigated whether the microbial population in CST-KO mice was altered. We found that the microbiome in CST-KO mice was indeed quite different in composition than its WT littermates (Fig. 6B-E&7). Most prominently, CST-KO mice showed a higher ratio of Firmicutes to Bacteroidetes (Fig. 6C-E), which is opposite to the decreased ratio observed in WT mice intra-rectally infused with CST [52], and supports the idea that a low ratio of Firmicutes to Bacteriodetes could be beneficial to dampen intestinal inflammation [52].

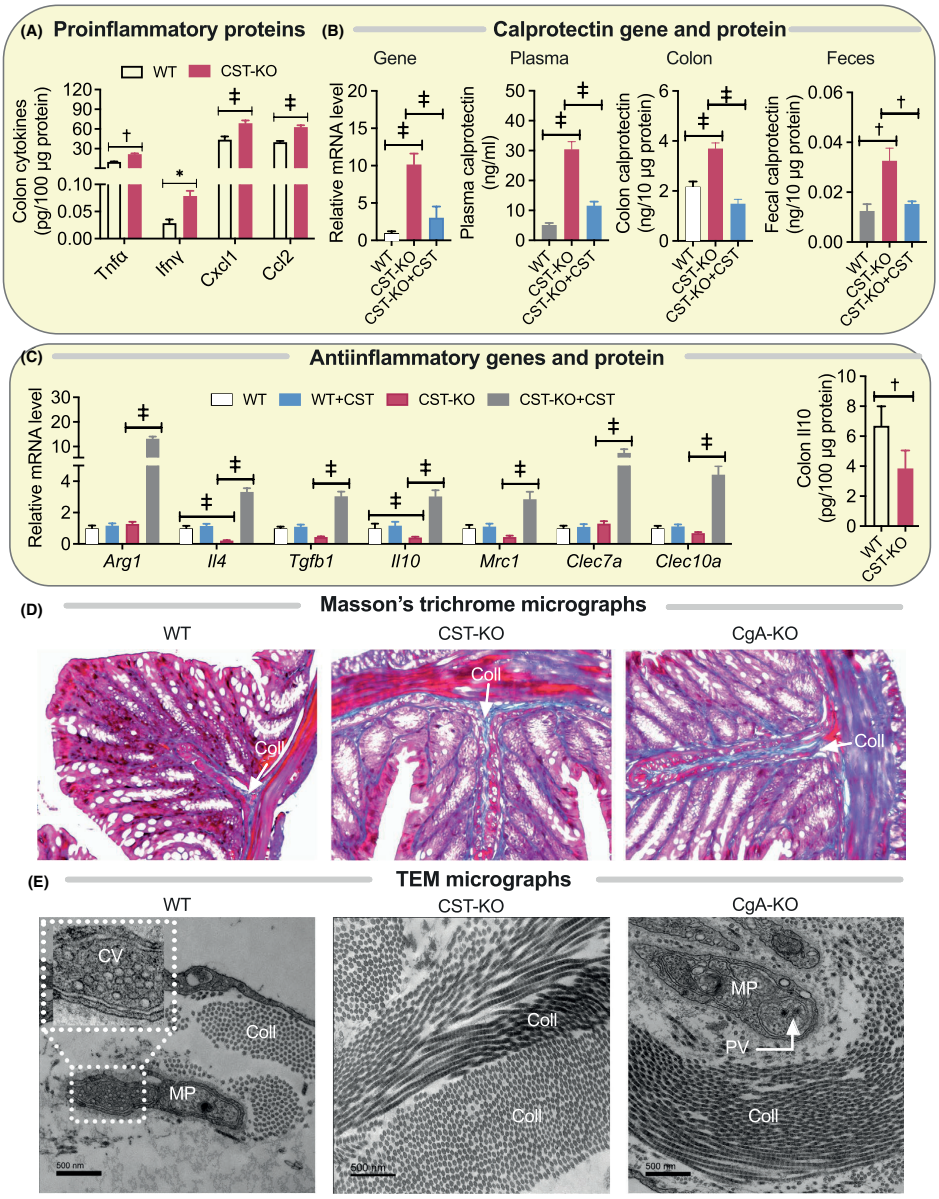

Fig. 5. Elevated levels of pro-inflammatory proteins and fibrosis in colon of CST-KO mice. (A) Increased expression of proinflammatory proteins (Tnfα, Ifnγ, Cxcl1, and Ccl2) in colon of CST-KO mice (n=8; 2-way ANOVA). **(B)** Increased expression of proinflammatory Calprotectin gene *s100A9* in colon and Calprotectin protein in plasma, colon, and feces of CST-KO mice, which were reversed upon administration of CST for 15 days (n=5; 1-way ANOVA). **(C)** Decreased expression of anti-inflammatory genes (*Arg1, Il4, Tgfb1, Il10, Mrc1, Clec7a,* and *Clec10a*) (n=8; 2-way ANOVA), and anti-inflammatory protein (Il10) (n=8; Welch's *t* test) in colon of CST-KO mice. CST treatment increased expression of anti-inflammatory genes in CST-KO mice. **(D)** Masson's trichrome-stained histological micrographs showing increased fibrosis in CST-KO and CgA-KO colon. **(E)** TEM micrographs showing increased presence of collagen fibers in CST-KO and CgA-KO mice. Coll, collagen; CV, clear cholinergic vesicles; MP, Meissner's plexus; PV, dense core peptidergic vesicles. *p<0.05; **p<0.01; ***p<0.001.

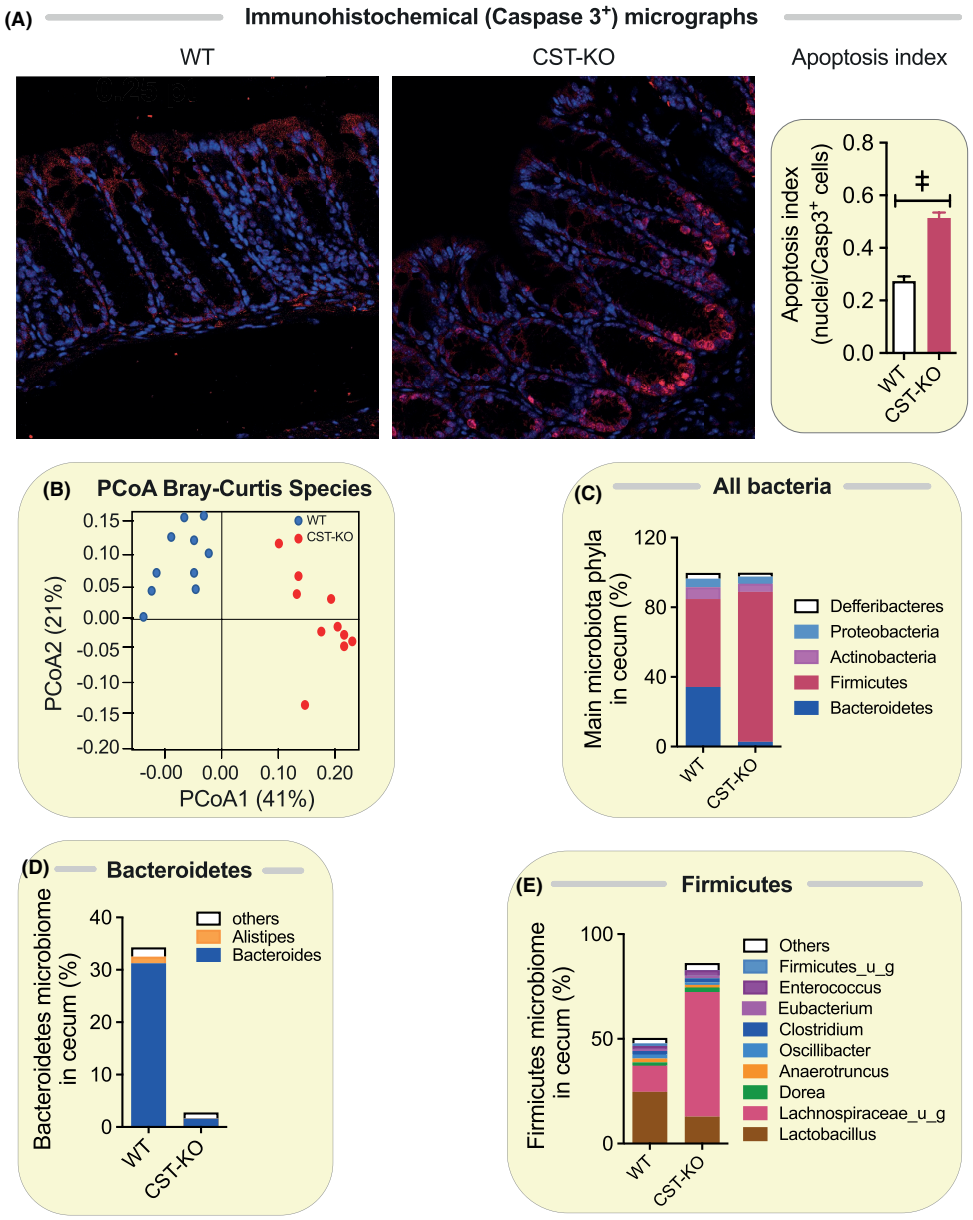

Fig. 6. Increased apoptosis and ratio of Firmicutes to Bacteriodetes in gut of CST-KO mice. **(A)** increased numbers of Caspase-3 positive apoptotic cells in colon of CST-KO mice. **(B)** Principal CoordiNATE Analysis (PCoA) of microbiome data using Bray-Curtis distance of species composition and abundance in individual WT (blue) and CST-KO (red) mice. **(C-E)** Microbiota composition of cecum of WT and CST-KO mice for main bacteria phyla **(C)** and the geni within the *Bacteriodetes* **(D)** and *Firmicutes* **(E).**

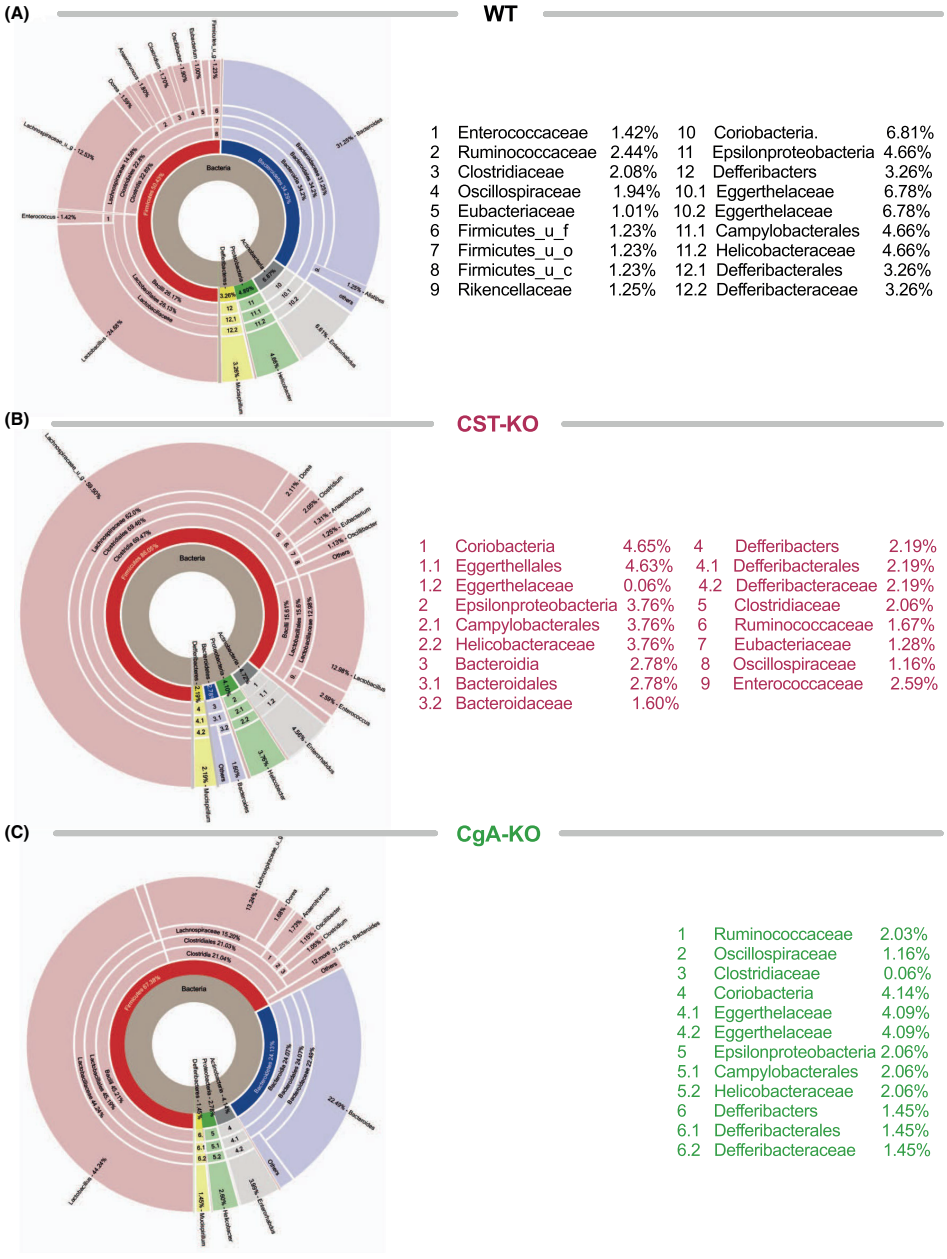

Fig. 7. Detailed microbial composition of WT, CST-KO, and CgA-KO mice. Graphs show complete microbiota composition in cecum of WT **(A)**, CST-KO **(B)**, and CgA-KO **(C)** mice. Circles from inside to outside show kingdom, phylum, class, order, family and genus.

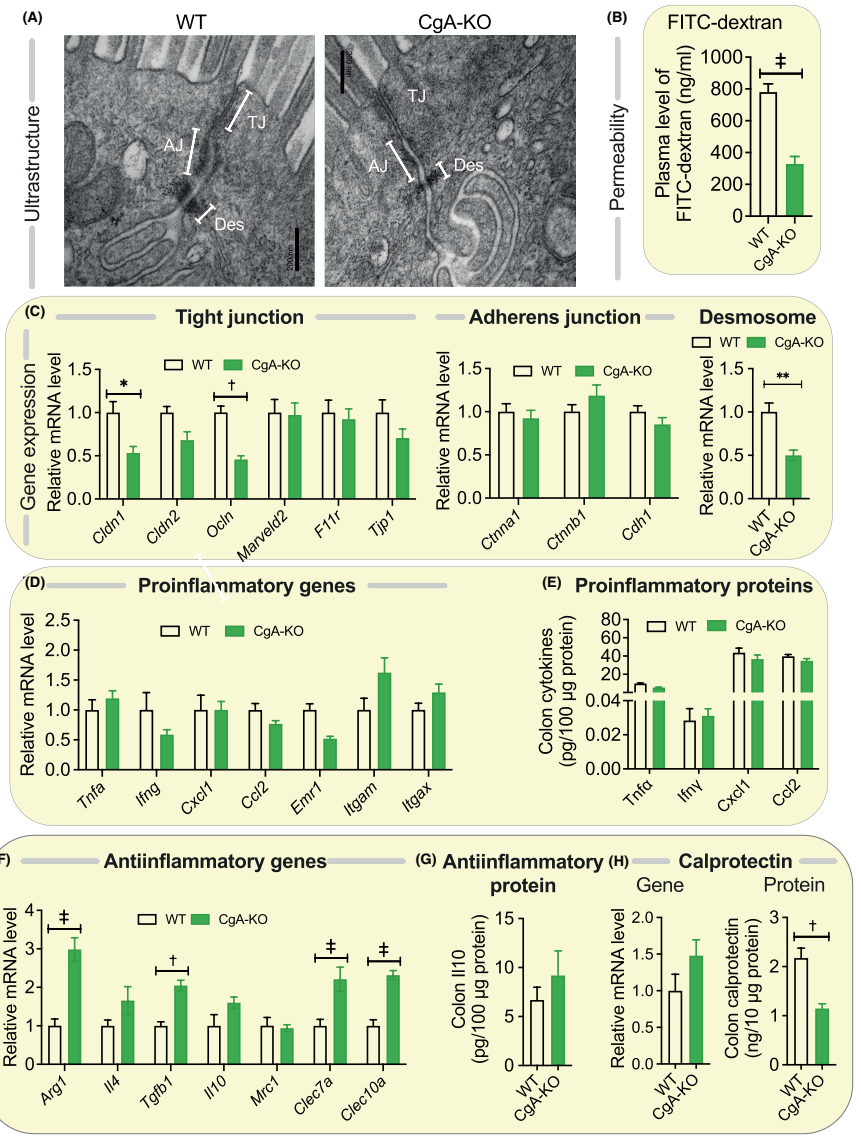

Fig. 8. Increased epithelial paracellular barrier function and lower inflammation in CgA-KO mice. **(A)** Electron microscopy micrographs of colon of WT and CgA-KO mice. TJ: tight junction. AJ: adherens junction. Des: desmosomes. **(B)** Gut permeability as measured by FITC-Dextran plasma level in WT and CgA-KO mice (n=7; Welch's *t* test). **(C)** Relative mRNA expression of genes in tight junction (*Cldn2*, *Ocln*, *Marveld2*, *F11r,* and *Tjp1*) (n=8, 2-way ANOVA), adherens junction (*Ctnna1, Ctnnb1*, and *Cdh1*) (n=8; 2-way ANOVA), and desmosome (*Dsg2*) (n=8; Welch's *t* test) in colon of WT and CgA-KO mice. Comparable expression of **(D)** proinflammatory genes (*Tnfa, Ifng, Cxcl1, Ccl2, Emr1, Itgam*, and *Itgax*), and **(E)** proinflammatory proteins (Tnfα, Ifnγ, Cxcl1, and Ccl2) in colon of WT and CgA-KO mice (n=8, 2-way ANOVA). Increased expression of **(F)** anti-inflammatory genes (*Arg1, Tgfb1, Clec7a, and Clec10a*) and comparable expression of anti-inflammatory genes (*Il4, Il10,* and *Mrc1*), and **(G)** anti-inflammatory protein (Il10) in colon of WT and CgA-KO mice (n=8; 2-way ANOVA or Welch's *t* test). **(H)** Comparable expression of *s100a9* gene and decreased expression of calprotectin protein in CgA-KO colon (n=5; Welch's *t* test). †, P<0.01; ‡, P<0.001.

That the leaky gut of CST-KO mice is inflamed and harbors dysbiotic luminal contents implies that CST is required for maintaining gut barrier integrity and preventing dysbiosis and gut inflammation. This is important because a "leaky gut" [53], dysbiosis [54], and hyperactivated immunity [55] are all well known to play a role in IBD. It is also consistent with others' observation that administration of exogenous CST can ameliorate colitis in mice [55].

CST is sufficient to tighten the leaky gut barrier in CST-KO mice and reduce inflammation

To test whether CST is sufficient to regulate gut permeability, we supplemented CST-KO mice with recombinant CST (intraperitoneal injections of 2 µg/g body weight once daily for at least 15 days), which reversed paracellular leakiness of FITC-dextran and the associated abnormalities such as: (i) reduced plasma FITC-dextran even lower than WT level (Fig. 2B), (ii) increased mRNA expression of *Ocln*, *Marveld2*, *F11r*, *Tjp1, and Dsg2* (Fig. 2F&H), (iii) decreased mRNA expression *of Cldn1, Cldn2*, *Tjp2, Tjp3, Ctnna1*, *Ctnnb1*, and *Cdh1,* as well as increased protein expression of Claudin-2 (Fig. 2F&G, 3A&C), (iv) decreased mRNA expression of proinflammatory genes (*Tnfa*, *Ifng*, *Cxcl1, Ccl2, Emr1*, *Il12b*, *Nos2*, *Itgam*, and *Itgax)* (Fig. 4G*)*, (v) increased expression of anti-inflammatory genes (*Arg1*, *Il4*, *Tgfb1*, *Il10*, *Mrc1*, *Clec7a*, and *Clec10a*) (Fig. 5C), (vi) decreased expression of Calprotectin gene (subunit *S100a9)* and protein levels (Fig. 5B). These findings demonstrate that CST reduces intestinal inflammation, and that CST is not only required for the integrity of the paracellular gut barrier, but is also sufficient to maintain the integrity of this barrier during homeostasis.

The paracellular permeability of the gut is intact in CgA-KO mice

Next, we carried out the gut barrier studies in CgA-KO mice, which not only lack CST, but also lack all other peptides derived from CgA. Ultrastructural studies showed no structural changes in TJ, AJ and desmosomes in CgA-KO mice compared to WT mice (Fig. 8A). To our surprise, we found that despite the absence of CST in these mice, the gut permeability for FITC-dextran was not increased (Fig. 8B); if anything, the paracellular barrier was even tighter than WT control mice. This decreased permeability was not accompanied by changes in CgA-KO mice in genes coding for components of TJ, AJ and desmosomes, except that *Ocln* and *Dsg2* gene expression were decreased in CgA-KO mice compared to WT mice (Fig. 8C). Likewise, proinflammatory gene and protein expressions were not affected in CgA-KO mice (Fig. 8D-E). In line with this, histology and EM revealed some fibrosis, but no excessive immune cell infiltration in the colon of CgA-KO mice (Fig. 5D&E). In contrast to the CST-KO mice, the CgA-KO mice even showed increased colonic expression of anti-inflammatory genes and proteins (Fig. 8F-G). The decreased colon levels of Calprotectin were also consistent

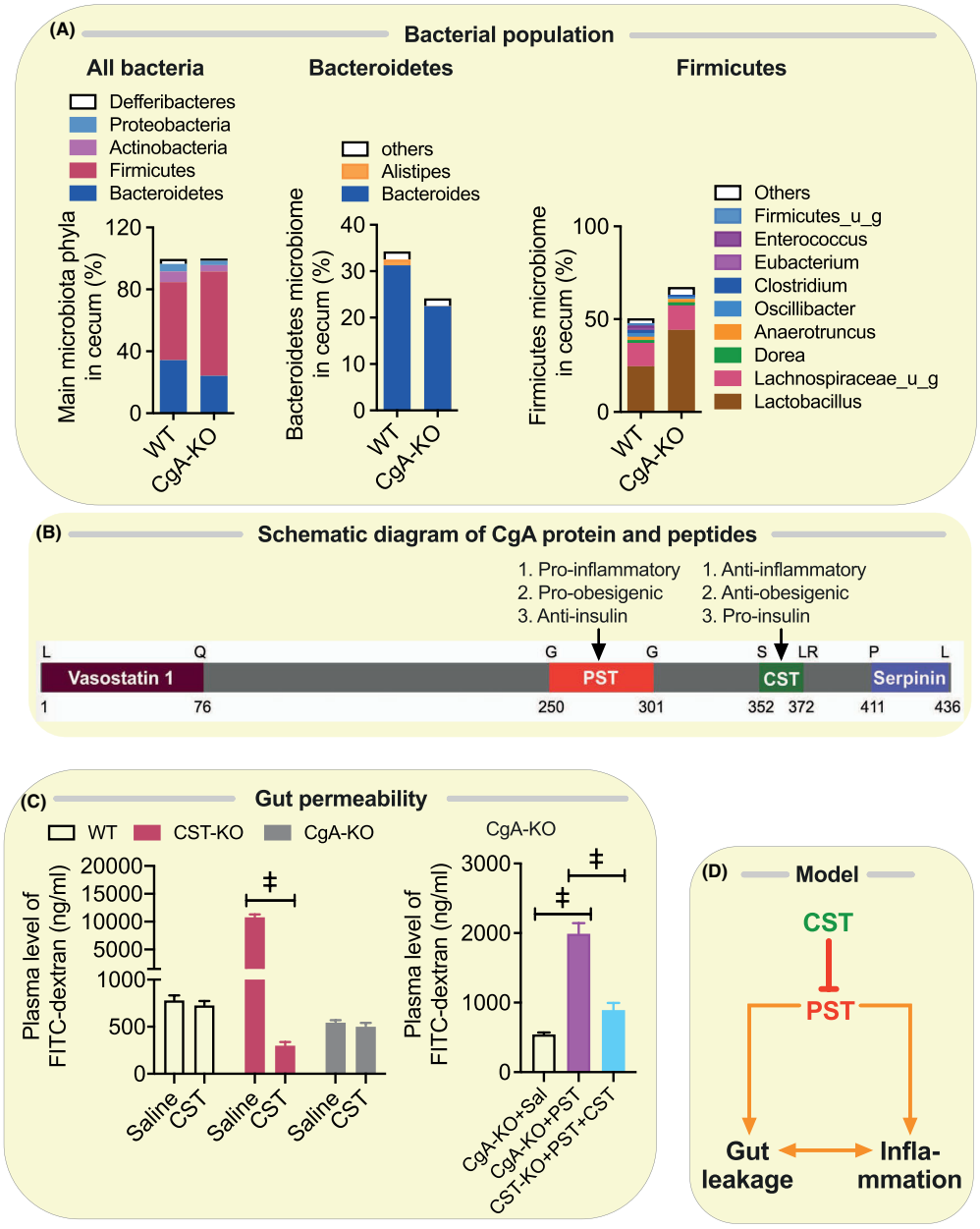

Fig. 9. Altered microbiome composition in CgA-KO mice and CST and PST oppositely regulate gut permeability. (A) Microbiota composition of cecum of WT and CgA-KO mice for main bacteria phyla and the geni within the Bacteriodetes and Firmicutes. **(B)** Scheme of CgA indicating the positions of the peptide products vasostatin 1, PST, CST and serpinin. **(C)** Gut permeability as measured by FITC-Dextran plasma levels in WT, CST-KO and CgA-KO mice treated with saline or CST (n=8; 2-way ANOVA) and in CgA-KO mice treated with PST or a combination of PST and CST (n=8; 1-way ANOVA). **(D)** Model of gut barrier regulation by CST and PST. ‡, P<0.001.

with decreased gut permeability (Fig. 8H). Compared to CST-KO mice, Firmicutes and Bacteroidetes bacteria populations in CgA-KO mice were more comparable to WT mice (Fig. 7&9A).

These findings suggest that CST is not essential in the absence of CgA. Because a leaky and inflamed gut was seen in the CST-KO mice which specifically lack the proteolytic fragment of CST, but not in CgA-KO mice which lack CST and all other peptides that are generated proteolytically from CgA, these findings raised the possibility that CST may be required to antagonize the actions of some other peptide hormone that is also a product of CgA.

Gut permeability is regulated by the antagonistic effects of two CgA peptides: CST and Pancreastatin (PST)

Among the various proteolytic peptides, we hypothesized that CST could be acting to balance the impact of PST, another peptide fragment of CgA (Fig. 9B). This hypothesis was guided by prior work [43] alluding to PST's deleterious impact on the gut mucosa, causing macrophage infiltration and inflammation. To test the nature of contribution of PST and CST on the gut paracellular barrier function, we supplemented the CgA-KO mice with either CST alone (2 µg/g body weight for 15 days), or PST alone (2 µg/g body weight for 15 days), or both PST and CST co-administered at equimolar ratio for 15 days. CST alone did not change leakiness of FITC-dextran in CgA-KO mice (Fig. 9C).

By contrast, PST alone increased the leakiness in CgA-KO mice and this leakiness was markedly reduced when the CgA-KO mice received both PST and CST at equimolar concentration (Fig. 9C). These findings indicate that PST increases the paracellular gut permeability, and that CST antagonizes this action. Together, these data lead us to propose a working model (Fig. 9D) in which the intestinal epithelial paracellular barrier is finetuned by the EECs via a major gut hormone, CgA. Our data show that CgA is proteolytically processed into two peptides, CST and PST, which tighten and loosen the gut barrier, respectively.

Discussion

The major finding in this work is the discovery of a molecular mechanism that involves a major EEC-derived gut hormone that has a balanced action on the paracellular gut barrier; two hormone derivatives of CgA, i.e., CST and PST, which either tighten or loosen the barrier, respectively. Because CgA-KO mice had unimpaired leakage of FITC-dextran, which was increased by exogenous administration of PST but not reduced by exogenous CST alone, findings also indicate that the barrier-homeostatic mechanism(s)

is contained within and converge at a molecular level on the prohormone CgA and its processing into counteracting peptide hormones. These findings are important because CST is a major peptide hormone in EEC which is proteolytically produced from CgA by proprotein convertase 1 (PC1) [55], cathepsin L [56], plasmin [40] and kallikrein [57]. Its abundance in the gut suggests that the permeability of the gut barrier is regulated by CST.

CgA is unique in having peptide domains that antagonize several functions including inflammation (CST suppresses while PST promotes), obesity (CST inhibits while PST stimulates) and sensitivity to insulin (CST increases while PST diminishes) [20,27,58]. Since CgA-KO mice lack both CST and PST, and we observed increased permeability in CST-KO mice and decreased permeability in CgA-KO mice, we reasoned that these opposing phenotypes are probably caused by the lack of PST in the CgA-KO mice. Indeed, the increased permeability in PST-treated CgA-KO mice and its reversal by co-treatments of PST and CST lead us to conclude that CST promotes the epithelial paracellular barrier function by counteracting PST. Nevertheless, the baseline gut permeability in CST-KO mice was higher than in CgA-KO mice supplemented with PST. This might suggest that there are other cleavage fragments of CgA that regulate the intestinal permeability or that the effects cannot be completely reversed.

While PST exerts deleterious impact on the gut mucosa, causing macrophage infiltration and inflammation [59], we found that lack of CST caused an increased expression of proinflammatory genes and proteins and treatment of CST-KO mice with CST reversed expression of those genes and proteins. These findings are in line the anti-inflammatory roles of CST [27,29] and with the low-grade systemic inflammation present in the CST-KO mice [27,29]. Our findings are also in congruence with the existing literature which showed increased TJ permeability in presence of increased proinflammatory cytokines such as TNF-α, IL-1β, and IFN-γ[60]. A reduced gut barrier function might facilitate the leakage of microbial components such as lipopolysaccharide (LPS) and peptidoglycan (PGN) from the lumen of the gut into the tissues, where they can bind to pattern recognition receptors and activate the immune system. In turn, the increased inflammation can result in further leakage of the gut. For instance, myosin light chain kinase (MLCK) plays critical roles in cytokine-mediated TJ regulation by disrupting the interaction between TJ proteins and the actin-myosin cytoskeleton [61] leading to damage of the TJ scaffold, which is crucial for the maintenance of barrier integrity [61]. Inflammatory cytokine-induced intestinal barrier loss is also believed to be due to endocytosis of TJ proteins, which also requires MLCK transcription and activity at the TJ [6], although we did not observe decreased protein levels of TJ proteins in the colon of CST-KO mice. In addition to the above mechanisms, TNF-α suppresses TJ barrier

function by decreasing expression of TJP1 protein, increasing expression of Claudin-2, and activating the NF-KB pathway [62]. IL-6 also increases expression of Claudin-2 [63]. In line with this, we observed elevated levels of Claudin-2 in the colon of CST-KO mice.

In addition to these indirect effects on TJ protein expression and structure via the immune system, it might also be possible that CgA and/or its cleavage products exert direct effects on the epithelial cells and thereby more directly regulate the TJs. To investigate this, it will be interesting to perform *in vitro* experiments, e.g., epithelial monolayers in perfusion chambers co-cultured with or without immune cells [64].

An interesting question is how CST and PST affect the AJs and desmosomes. Not only TJ components, but also desmosome and AJ components are altered in CST-KO mice and these changes are reversible by administration of exogenous CST. It might be possible that the AJs and/or desmosomes are part of a feedback mechanism to compensate the altered TJ structure. Previously, by using Dsg2-KO mice, desmosomes have been shown to regulate the intestinal barrier [45]. Moreover, Dsg2 modulates the production of glial cell-derived neurotrophic factor (GDNF) in inflammatory bowel disease patients [65]. Therefore, a possible scenario is that CgA-derived peptides regulate the release of GDNF from enteric glial cells in the gut wall, which in turn affects the epithelial junctions.

The present study unequivocally demonstrates that CST is a key regulator of paracellular gut permeability since the absence of CST (CST-KO) is accompanied by dysbiosis, increased gut permeability for FITC-dextran, increased infiltration of macrophages and $CD4^+$ T cells, and higher local inflammatory gene and protein expression with the substitution of CST-KO mice with CST reversing many of the above abnormalities. Do gut hormones simulate the actions of CST? While Cholecystokinin (CCK) decreases mucosal production of proinflammatory cytokines induced by LPS and increases the expression of seal forming TJ proteins (OCLN, Claudins and JAM-A) [66], Ghrelin ameliorates sepsis-induced gut barrier dysfunction by activation of vagus nerve via central ghrelin receptors [53] as well as by decreasing production of TNF-α and IL-6 [67]. Glucagon-like peptide 1 (GLP-1) has also been shown to decrease inflammation of enterocytes [68]. Vasoactive intestinal peptide, a neuropeptide found in lymphocytes [69], mast cells [70], and enteric neurons of the gastrointestinal tract regulates both epithelial homeostasis [71] and intestinal permeability [72]. The multitude of effects of CST on gut physiology and their recapitulation by these well-investigated gut hormones establishes CST as an important gastrointestinal peptide hormone.

CST-KO mice (loss of barrier integrity) are hypertensive and insulin-resistant on normal chow diet [27,38] and CgA-KO mice (intact barrier integrity) have a normal blood pressure and are supersensitive to insulin in both normal chow and on high fat diet [18,20]. Could metabolic endotoxemia explain the above phenotypes? Besides gastrointestinal peptide hormones and neuropeptides, TJ homeostasis is altered by proinflammatory cytokines, pathogenic bacteria, and pathological conditions like insulin resistance and obesity. Firmicutes (e.g., *Lactobacillus*, *Clostridium*, and *Enterococcus*) and Bacteroidetes (e.g., *Bacteroides*) constitute the major bacterial phyla in the gut [73]. Toxigenic bacteria cause diarrhea *via* increased ion permeability of the pore pathway [51], but gut microbes might also affect the leak pathway. Enteric pathogenic bacteria such as *Escherichia coli* and *Salmonella typhimurium* alter the intestinal epithelial TJ barrier and cause intestinal inflammation [74]. LPS alters expression and localization of TJ proteins such as TJP1 and OCLN [75]. Therefore, it would be interesting to study whether the systemic LPS levels support the observed dysbiosis-related loss of gut barrier in the CST-KO mice. Since high fat diet-induced insulin-resistant mice and patients with obesity or diabetes display elevated levels of Firmicutes and Proteobacteria compared to the beneficial phylum Bacteroidetes [76], it is conceivable that dysbiosis (i.e., increased Firmicutes and decreased Bacteroidetes) in CST-KO mice is due to their resistance to insulin. This is further supported by the fact that *Akkermansia muciniphila*, which regulates intestinal barrier integrity, is less abundant in diabetic individuals [77]. Furthermore, the daily administration of *A. muciniphila* is known to mitigate high fat-induced gut barrier dysfunction [78]. Conversely, metabolic endotoxemia, resulting from the loss of barrier integrity, contributes to insulin resistance and obesity [76,79]. The intact paracellular barrier function in CgA-KO mice could thus be due to their heightened sensitivity to insulin.

Human calprotectin, a 24 kDa dimer, is formed by S100A8 and S100A9 monomers and released from neutrophils and monocytes. In fact, this calprotectin complex constitutes up to 60% of the soluble proteins in the cytosol of neutrophils [80] and fecal calprotectin levels serve as a marker of intestinal inflammation [81,82]. Because of the positive correlation between calprotectin concentration in gut lavage fluid and intestinal permeability [83], calprotectin levels are also used to assess intestinal permeability. Increased calprotectin levels have been reported to increase intestinal permeability [49]. Therefore, increased gene and protein expressions in the colon of CST-KO mice are in line with the increased permeability in CST-KO mice.

Elevated plasma CST levels in patients suffering from Crohn's disease both in disease remission and flare-up as compared to elevated precursor CgA in Crohn's disease flare-ups only suggests that while CgA is generally upregulated in Crohn's disease, it is only efficiently processed to CST during disease remission. Previous studies revealed higher

plasma CgA levels during flare-ups as compared to ulcerative colitis and Crohn's disease patients in remission [34]. An open question remains how the levels of PST and CST are regulated in ulcerative colitis and Crohn's disease patients. For CST our findings suggest a proteolytic switch, where CgA and CST are excessively produced in Crohn's disease regardless of the disease state, but CgA is converted more into the anti-inflammatory CST in quiescent Crohn's disease. The gut barrier dysfunction in CST-KO mice is reminiscent of active Crohn's disease, because the gut permeability provided by cell-cell junctions is generally diminished in IBD patients [84]. Based on our findings, we hypothesize that the higher levels of CST during Crohn's disease remision promote anti-inflammatory responses and aid restoration of epithelial cellular junctions.

Material and Methods

Mice. Male wild type (WT), CST-knockout (KO) and CgA-KO mice (3 weeks old) on C57BL/6 background were kept in a 12 hr dark/light cycle on normal chow diet (NCD: 13.5% calorie from fat; LabDiet 5001, Lab Supply, Fort Worth, TX). Experiments were conducted in a blinded fashion whenever possible. For terminal procedure, mice were deeply anesthetized (assessed by pinching toe response) with isoflurane followed by harvesting of tissues. Euthanasia was achieved through exsanguination. For rescue experiments with exogenous CST and PST, mice were injected intraperitoneally with CST (2 µg/g body weight) and/or PST (2 µg/g body weight) at 9:00 AM for 2-4 weeks before collecting feces or harvesting tissues. All mouse studies were approved by the UCSD and Veteran Affairs San Diego Institutional Animal Care and Use Committees and conform to relevant National Institutes of Health guidelines.

Gut permeability assay. Fluorescein isothiocyanate conjugated dextran (FITC-dextran-FD4, 4 kDa; MilliporeSigma, St. Louis, MO) was administered (44 mg/100 g body weight) to 8 hr fasting mice by oral gavage followed by collection of blood after 4 hr of administration from the heart under deep isloflurane anesthesia [85]. Plasma concentration of FITC was determined by comparison of fluorescence signals with a FITC-dextran standard curve.

Measurement of tissue cytokines, CST, CgA and calprotectin. A portion of the colon was homogenized in PBS and cytokines were measured in 20 µl of the homogenate using U-PLEX mouse cytokine assay kit (Meso Scale Diagnostics, Rockville, MD) via the manufacturer's protocol. Mouse EIA kits from CUSABIO (CUSABIO Technology LLC, Houston, TX) were used to determine CgA (CSB-EL005344MO), CST (CSB-E17357m), and calprotectin (CSB-EQ013485MO).

Determination of CgA and CST levels in human plasma. EDTA-plasma was collected from patients with Crohn's disease (N=89) and ulcerative colitis (N=101) (Supplemental table S1) and healthy controls (N=50). Intestinal inflammation was assessed by colonoscopy. A flare-up was defined as histological and endoscopic disease activity, whereas remission entailed absence of endoscopic inflammation and histological disease activity. EDTA-plasma from healthy controls (Supplementary table S2) was obtained from the Mini Donor Service at the University Medical Center Utrecht. Samples were thawed and diluted 50-times with extraction buffer (PBS with 0.5% Tween and 0.05% sodium azide), thoroughly vortexed followed by incubation at 4°C for 20 min on a rolling device. Afterwards samples were centrifuged at 760 ×g for 20 min at 4°C and the supernatant was collected for analysis. Samples were collected in compliance with the Declaration of Helsinki. EDTA plasma was used to quantify CgA and CST using ELISA (CUSABIO CSB-E17355h, CSB-E09153h). Informed consent was obtained, and the study was conducted in accordance with the Institutional Board Review of the University Medical Center Utrecht (approval number NL35053.041.11 & 11-050).

Colon histological stains and immunohistochemistry. Mouse colon was isolated, washed in PBS and dehydrated in ethanol series, xylene and paraffin. Afterwards, the tissue was embedded in paraffin, formalin fixed, and paraffin embedded (FFPE) and sections of 5 µM were cut. Hematoxylin and eosin (HE) and Masson's trichrome (MT) staining were performed using standard techniques. For the immunohistochemistry, the tissue was heated in citrate buffer (pH 6.0, 1.0 nM citric acid) and endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol. Standard indirect immunoperoxidase procedures were used for detection (Vectastain and SK-4800, Vector Laboratories) with the antibody Rabbit-αKi-67 (1:100, Novus Biologicals), Rat-αF4/80 (1:500, Caltag laboratories and slides were counterstained with Mayer's haematoxylin. Followed by mounting using Quick D mounting medium. For fluorescent staining, slides were incubated with Rabbit-αCASPIII antibody (1:100, ab2302, Abcam) and nuclear counterstain with DAPI using Fluoromount-G (0100-01, Southern Biotech). Slides were imaged using the PerkinElmer Vectra (Vectra 3.0.3; PerkinElmer, MA) at 20x magnification or imaged using the Leica SP8 with a water dipping objective.

Transmission Electron Microscopy (TEM) and morphometric analysis. To displace blood and wash tissues before fixation, mice were cannulated through the apex of the heart and perfused with a Ca^{2+} and Mg^{2+} free buffer composed of DPBS and 9.46 mM KCl as described [73]. Mouse and human colon fixation, embedding, sectioning and staining were done as described [73]. Grids were viewed using a JEOL JEM1400-plus TEM (JEOL, Peabody, MA) and photographed using a Gatan OneView digital camera with 4×4k resolution (Gatan, Pleasanton, CA). Morphometric analyses of the dense core

vesicles were determined as described [86]. TJ length and diameter were determined by measuring the lengths and perpendicular widths using the line tool in NIH ImageJ software.

Tight junction protein analysis via immunoblotting. Mouse colon pieces were homogenized in a buffer containing phosphatase and protease inhibitors, as previously described [19]. Colon lysates were subjected to SDS-PAGE and immunoblotted. Followed by staining with Rabbit-αOccludin (1:1000, ab216327, Abcam, Cambridge, MA, USA), Rabbit-αClaudin 1 (1:2000, ab180158, Abcam), Rabbit-αClaudin 2 (2μg/ml, 51-6100, Thermo Fisher Scientific, Waltham, MA, USA), Mouse-αE-cadherin (1:500, 610182, BD Biosciences, San Jose, CA, USA), Rabbit-αcatenin (1:1000, C2081, Sigma-Aldrich, St. Louis, MO, USA) or Rat-αZO-1 (1:500, R26.4C, DSHB, Iowa City, Iowa, USA).

Flow cytometry analysis. Isolated colon cells from digested mouse colon were stained with fluorescence-tagged antibodies to detect macrophages ($CD45^+CD11b^+Emr1^+$) and helper T cells ($CD45^+CD4^+$). Data were analyzed using FlowJo.

Microbiome analysis. DNA was extracted from cecum of WT, CST-KO and CgA-KO (normal chow diet (NCD), littermates) using the Zymobiomics miniprep kit. Isolated DNA was quantified by Qubit (Thermo Fisher Scientific). DNA libraries were prepared using the Illumina Nextera XT library preparation kit. Library quantity and quality was assessed with Qubit and Tapestation (Agilent Technologies). Libraries were sequenced on Illumina HiSeq platform 2×150bp. Unassembled sequencing reads were directly analyzed by CosmosID bioinformatics platform (CosmosID) [87] for multi-kingdom microbiome analysis and profiling of antibiotic resistance and virulence genes and quantification of organism's relative abundance. This system utilizes curated genome databases and a high-performance data-mining algorithm that rapidly disambiguates hundreds of millions of metagenomic sequence reads into the discrete microorganisms engendering the particular sequences. Data mining, interpretation and comparison of taxonomic information from the metagenomic datasets were performed using Calyspo Bioinformatics software V.8.84.

Real Time PCR. Total RNA from colon tissue was isolated using RNeasy Mini Kit and reverse-transcribed using qScript cDNA synthesis kit. cDNA samples were amplified using PERFECTA SYBR FASTMIX L-ROX 1250 and analyzed on an Applied Biosystems 7500 Fast Real-Time PCR system. Primer sequences are in Supplemental table S3. All PCRs were normalized to *Rplp0*, and relative expression levels were determined by the $\Delta\Delta C_t$ method as described [24].

Statistics. Statistics were performed with PRISM 8 (version 8.4.3; San Diego, CA). Data were analyzed using either unpaired two-tailed Student's *t*-test for comparison of two groups or one-way or two-way analysis of variance for comparison of more than two groups followed by Tukey's *post hoc* test if appropriate. All data are presented as mean ± SEM and significance were assumed when $p<0.05$.

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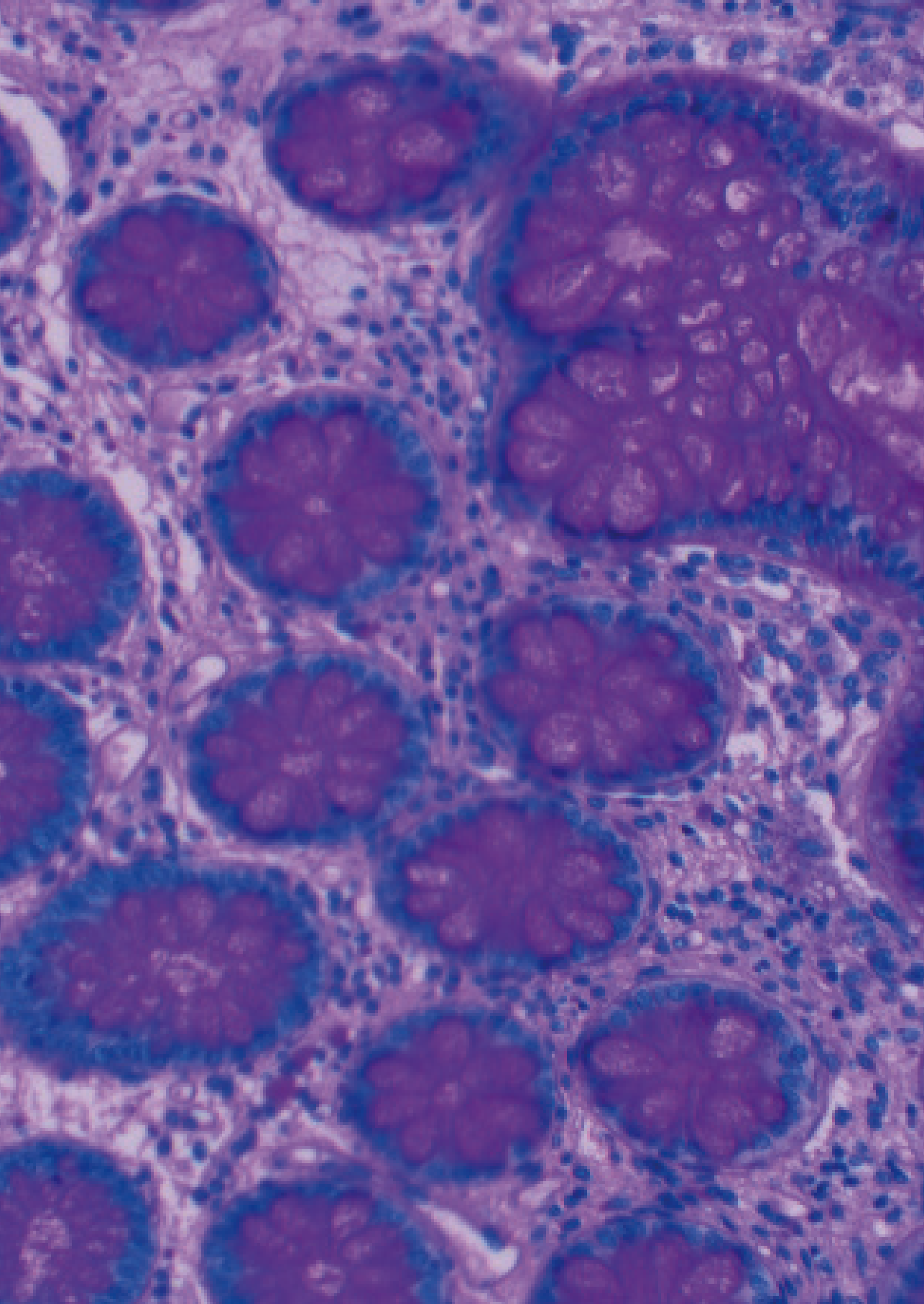

Chapter 3

Catestatin regulates the colonic mucus layer in inflammatory bowel disease

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Abstract

Background The pro-hormone chromogranin A (CgA) and its bioactive cleavage product catestatin (CST) are both associated with inflammatory bowel disease (IBD) and dysregulated barrier functions, but their exact role has remained elusive. Here, we demonstrate that CST regulates the colonic mucus layer.

Methods CST levels were measured in feces of IBD patients. The mucus layer, goblet cells, and immune cell infiltration were analyzed by histology and electron microscopy in colon tissue from IBD patients and mice with selective deletion of the CST-coding region of the CgA gene.

Results CST levels were elevated in feces of IBD patients compared to healthy controls. The thickness of the mucus layer was increased in non-affected, but not in inflamed, regions of the colon in IBD patients. The thickness of the mucus layer and concomitant mucus production were also increased in the CST-KO mouse. This mucus phenotype in CST-KO mice could be reversed by bone marrow transplantation from wildtype mice.

Conclusions CST produced by bone-marrow derived immune cells reduces production of the mucus layer in the intestine. This might contribute to the reduced mucus layer in inflamed colon regions of IBD patients. Additionally, CST feces levels might be a biomarker for IBD.

Introduction

Approximately two million people in the United States suffer from IBD, comprising Crohn's disease (CD) and ulcerative colitis (UC)(1). In IBD patients the plasma levels of both the pro-hormone chromogranin A (CgA)(2–6) and its bioactive cleavage product catestatin (CST: human $CgA_{352-372}$)(6–8) are increased. CST is generally considered anti-inflammatory(9,10) and treatment of UC mouse models with CST was found to reduce macrophage infiltration of the colon and attenuate inflammation(11–13). We recently showed that CST-KO mice, with a selective deletion of the CST-coding region of the *Chga* gene, display severely compromised intestinal permeability and inflammation in the colon(6). Additionally, the colon of CST-KO mice displays increased fibrosis, macrophage infiltration and a bacterial population reminiscent of IBD and/or inflammatory bowel syndrome patients(6). These inflammatory and permeability phenotypes are reversible by administration of exogenous CST(6). However, the exact role of CST in mucus regulation has not yet been addressed.

In addition to the epithelial cellular junctions, the mucus layer contributes to the protective barrier function of the colon(14). The mucus is produced by the goblet cells and consists of antimicrobial enzymes, immunoglobulins, glycoproteins and mucins (MUC)(14), with the latter two components defining the mucus layer composition. MUC2 is particularly important for mucus organization in the colon, since it establishes the connection between the compact inner layer and outer, more loose, mucus layer(15). The outer layer interacts with bacteria, while the inner layer is impenetrable and protects the mucosa from bacterial infiltration(15). A damaged mucus layer exposes the intestinal epithelial cells to gut microbial components which might lead to colonic inflammation(14). In UC, the mucus barrier is damaged independently of local inflammation, suggesting an intrinsic defect of the mucus layer(16). Mucus production is generally less affected in CD, but patients still display bacterial infiltration, again indicating that the mucus composition is not sufficient to maintain (mucosal) homeostasis(14,17).

Aim of this study was to identify how CST affects the colonic mucus layer. We characterized the thickness of the mucus layer in non- and inflamed regions of the colon of CD patients, inflamed colon of UC patients, and healthy controls. Moreover, we measured the CST concentrations in feces of IBD patients. We also characterized the thickness of the mucus layer in CST-KO and wildtype (WT) mice. Since we recently showed that macrophages (and possibly other bone-marrow derived immune cells) are major producers of CST(18,19), we determined the effect of bone-marrow transfer from WT mice into CST-KO mice on the colonic mucus layer.

Results

Feces CST correlates with IBD activity

We recently showed that CST is elevated in plasma of CD patients regardless of disease state, flare-up or remission, compared to healthy controls(6). Here, we addressed whether CST is also elevated in the feces of IBD patients. We measured CgA and CST levels in feces samples from a cohort of long-standing CD and UC patients stratified for inflammation by colonoscopy and confirmed by calprotectin levels (Fig. S1A-B, Table S1). For CD, CgA feces levels were only increased in patients with flare-ups, whereas for both CD and UC, CST feces levels were increased regardless of disease state (Fig. 1, Table S1). These findings are reminiscent to our previous findings in plasma(6) and support the conclusion that CST is not only elevated in circulation, but also locally in the colon of IBD patients.

Mucus layer thickness depends on local inflammation

In order to address the relation between inflammation and the mucus layer, we compared non-inflamed and inflamed regions of colon biopsies of IBD patients (Fig. 2A-B, Fig. S2). Using cytochemistry and electron microscopy, we observed an increased thickness of the mucus layer at non-inflamed sites of the colon in IBD patients, while the thickness in inflamed regions was unaltered compared to a healthy individual (Fig. 2C-G). Moreover, electron microscopy revealed that the microvilli were shorter in inflamed as compared to non-inflamed regions of the colon within the same patients (Fig. 2C-F). Especially for CD patients, non-inflamed regions displayed longer microvilli and increased mucus thickness (Fig. 2C-E, Table S2). The thickness of the protective mucus layer of CD patients thus depends on the inflammatory state of the mucosa.

CST affects goblet cells and promotes mucus production

In the next set of experiments, we addressed the question whether the altered mucus layer in IBD patients could be linked to elevated levels of CST. In the colon of 3 months old CST-KO mice, the thickness of the mucus layer and the length of the microvilli were increased when compared to WT mice (Fig. 3A-C). The number of goblet cells was also increased in the colon of CST-KO mice (Fig. 3D-E), and electron microscopy revealed an altered morphology of these goblet cells (Fig. 3F-H). Quantitative PCR showed that expression of MUC2 was increased in the CST-KO mice (Fig. 3I). This increased MUC2 expression was fully reversible by intraperitoneal injection of CST (Fig. 3I).

Bone-marrow derived immune cells produce CST in the colon

We recently showed that macrophages are major producers of CST and that macrophage-produced CST is both sufficient and required for normalization of the blood pressure in CST-KO mice(19).CST-KO mice are hypertensive due to increased

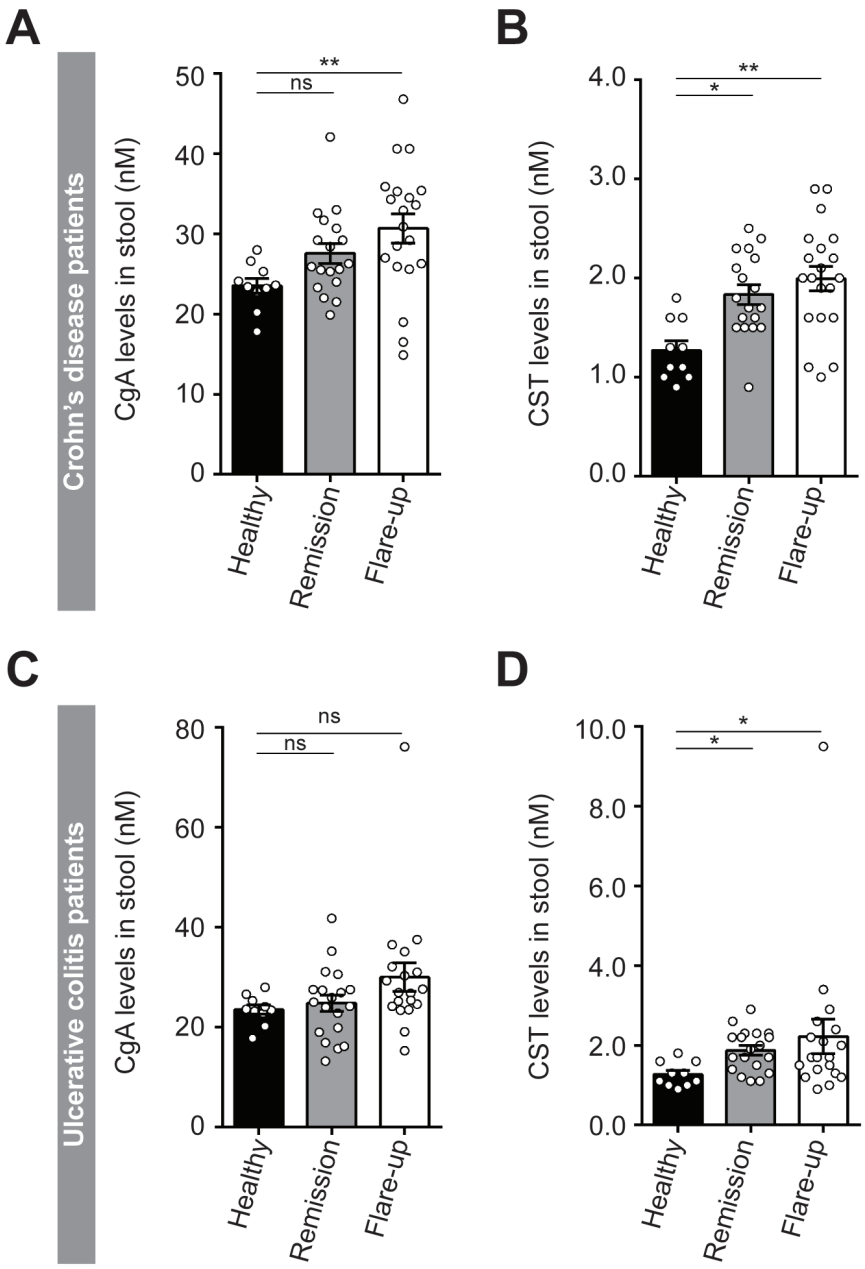

Fig. 1. CST correlates with IBD activity. (A) CgA levels in feces of healthy donors (black) and Crohn's disease (CD) patients in remission (grey) or flare-up (white). **(B)** Same as panel A, but now for CST levels in feces. **(C)** Same as panel A, but now CgA levels for Ulcerative Colitis (UC) patients. **(D)** Same as panel C, but now for CST. Kruskal-Wallis *: P<0.05; **: P<0.01; ***P<0.001.

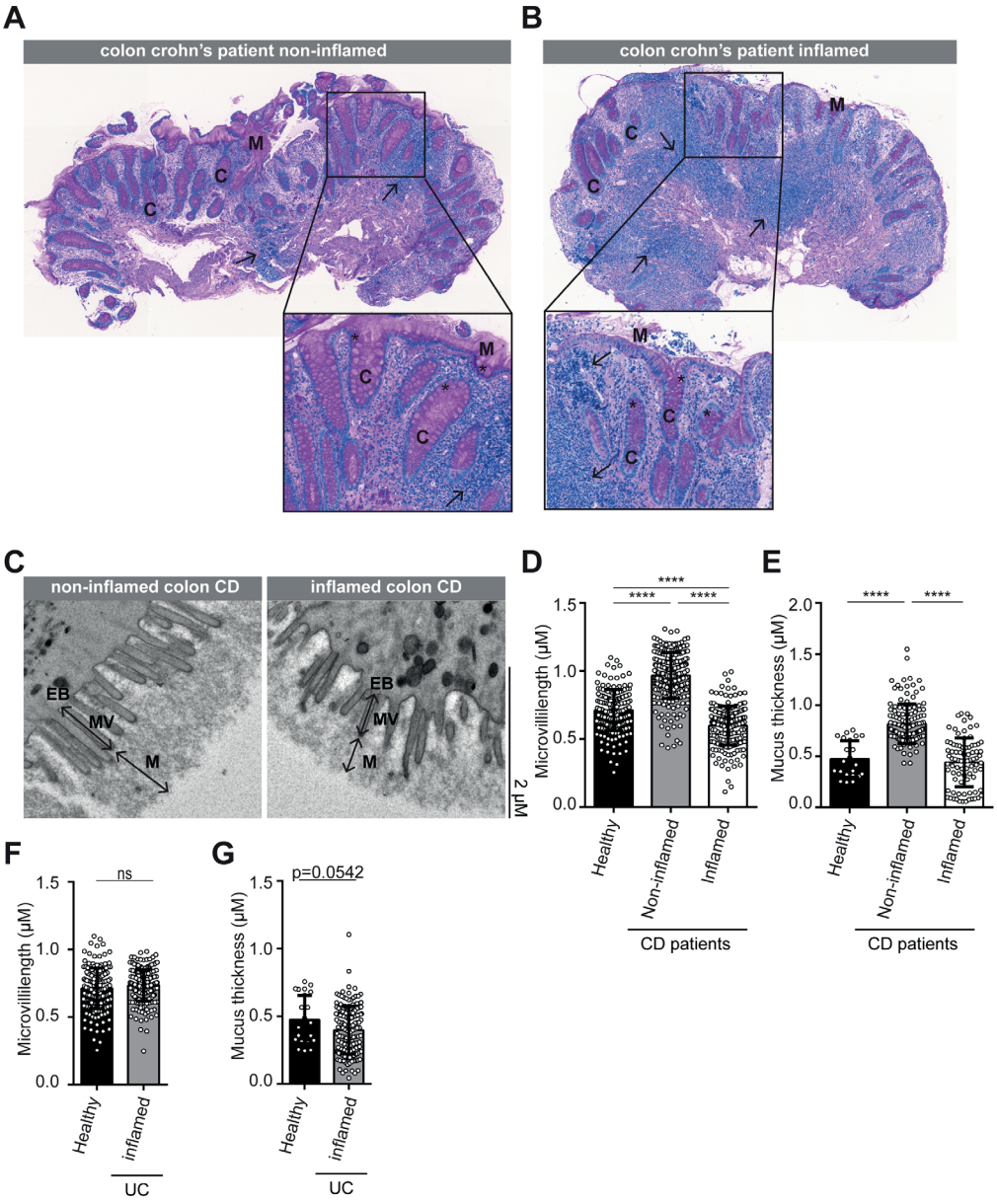

Fig. 2. IBD inflammation links to mucus layer thickness. (A-B) Representative images of periodic acid–Schiff (PAS) stained colon cross-sections of non-inflamed **(A)** and inflamed **(B)** colon tissue of Crohn's disease (CD) patients with enlargement in black framework. M: mucus, C: crypt, *: goblet cell and arrows indicate immune cell infiltration. **(C)** Representative electron microscopy (EM) micrographs of colon showing mucus layer (M), microvilli (MV) and epithelial border (EB). **(D-G)** Quantification of microvilli length and mucus thickness in colon of Crohn's disease (CD) **(D-E)** and Ulcerative Colitis (UC) **(F-G)** patients. Kruskal-Wallis *: P<0.05; **: P<0.01; ***P<0.001; ****P<0.0001.

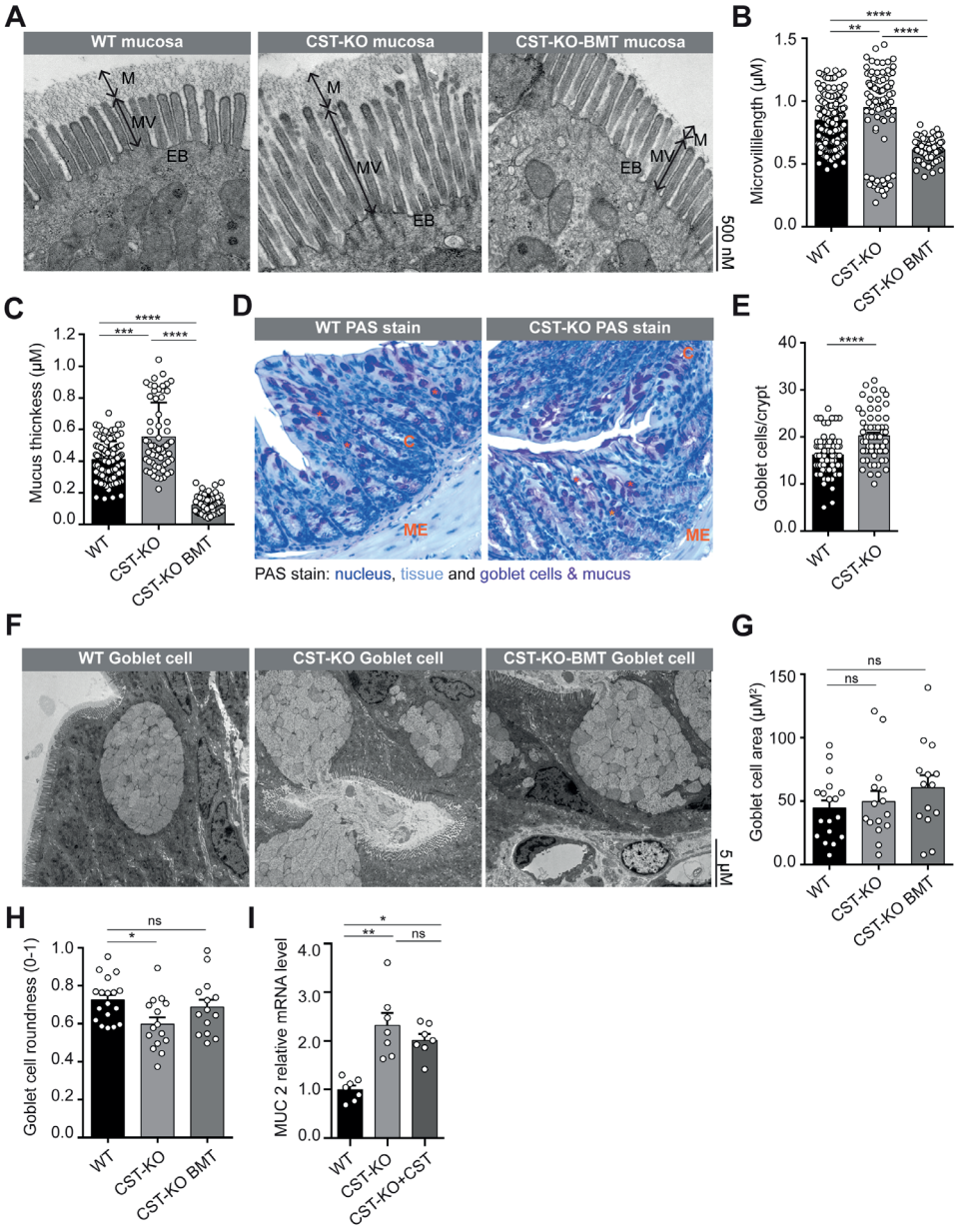

Fig. 3. CST affects goblet cells and mucus production. (A) Representative electron microscopy (EM) micrographs of colon from wildtype (WT), CST-KO and CST-KO mice that received a bone marrow transplant from WT mice (CST-KO-BMT). Images show mucus layer (M), microvilli (MV) and epithelial border (EB). **(B-C)** Quantification of microvilli length **(B)** and mucus thickness **(C)** from panel A. **(D)** Representative images of periodic acid–Schiff (PAS) stained colon cross-sections of WT and CST-KO mice. ME: muscularis externa, M: mucus, C: crypt, *: goblet cell. **(E)** Quantification of goblet cells per crypt from panel D. **(F)** Electron microscopy micrographs of colon showing goblet cells. **(G-H)** Quantification of goblet cell area **(G)** and circularity **(H)** from panel F. **(I)** Relative mRNA level of *MUC2* for WT, CgA-KO, CST-KO and CST-KO treated with CST. Kruskal-Wallis *: P<0.05; **: P<0.01; ***P<0.001; ****P<0.0001.

production of catecholamines, and this hypertensive phenotype could be fully reversed by depletion of macrophages or by bone-marrow transfer from WT mice to CST-KO(19). Bone-marrow transfer in CST-KO mice was also found to restore the phenotype of the mucus layer by reducing its thickness and the length of the microvilli (Fig. 3A-C). These results indicate that CST produced by macrophages (or other bone-marrow immune cells) regulates the colonic mucosa.

Discussion

In this study, we showed that CST is increased in the feces of IBD patients regardless of disease activity, whereas feces levels of its precursor CgA are only increased in CD patients with active disease. We also found that, compared to a healthy control, the thickness of the mucus layer and the length of the microvilli are increased in non-inflamed colon regions of CD patients, whereas the mucus layer is unaltered and the microvilli are shorter in inflamed regions. CST-KO mice display an increased thickness of the colonic mucus layer as well, in line with the increased presence and altered morphology of the goblet cells and increased levels of transcription of Muc2, a major component of the mucus layer(15). These microvilli and mucus phenotypes could be fully reversed by bone-marrow transfer from WT cells. From these findings, three important conclusions can be drawn:

First, not only blood levels(6), but also feces levels of CgA and CST might be markers for disease severity of IBD, particularly CD. CST levels are increased both in plasma(6) and feces (this study) of IBD patients irrespective of disease activity, whereas levels of CgA, the precursor of CST, are only elevated during active CD. These findings suggest a proteolytic switch, where CgA and CST are excessively produced in IBD regardless of the disease state, but CgA is cleaved more into CST in quiescent disease. As we recently reported, CST inhibits colon inflammation and promotes the intestinal epithelial barrier function by counteracting pancreastatin, another cleavage product of CgA(6). Thereby, the elevated CST levels in IBD patients are suggestive of a feedback mechanism to counteract the disease state and might help to normalize the colon function(6).

Second, we found that the mucus layer thickness and microvilli length in IBD patients depend on local disease activity in the colon. Between 1970 and 1980, several studies showed increased goblet cell numbers in the ileum and colon(20,21) and increased mucus secretion in the ileum of CD patients(21). In contrast, UC patients were characterized with decreased goblet cell presence and glycocalyx (inner mucus layer)(20,22,23). These outcomes are based on case reports and did not include non-

inflamed intestine of the same patient. In our study, non-inflamed colon regions of CD patients were characterized with a thicker mucus layer than inflamed colon regions from the same patient, whereas we did not observe significant differences for UC patients. Since the patient biopsies were taken only a few centimeter apart, this indicates tight local mucosal regulation. Moreover, the mucus layer in non-affected colon regions of CD patients was even thicker than for healthy controls, indicating excessive mucus production, possibly as a compensatory mechanism to protect the colon from microbial infiltration. The length of the microvilli was affected in the same way: increased in non-inflamed tissue and shortened in inflamed colon tissue, a pattern observed before in pigs(24). Previously, electron microscopy studies reported destroyed colon microvilli in UC patients(20,23), whereas no consistent changes were found in CD patients(20,22). Moreover, in the ileum of CD patients, a decreased microvilli density has been reported, as well as downregulation of genes related to microvilli formation, F-actin bundling, membrane cytoskeleton cross-linking and intermicrovillar adhesion complex(25). Our data add to this and show that changes of the mucus layer and microvilli in the colon depend on the local inflammation state in CD patients.

Third, we conclude that CST produced by bone-marrow derived cells regulates the production of the protective mucus layer. We found that the thickness of the mucus layer in the colon of CST-KO mice is increased, in line with the increased infiltration of immune cells reported previously(6)[6]. It seems contrasting that CST presence reduced the protective mucus layer, but at the same time reduces intestinal permeability as we showed previously(6). So far, we don't know whether the composition of the decreased mucus layer in presence of CST would be more protective against bacterial infiltration. Another open question is whether the effects on mucus regulation might be caused by CST alone or by the balance between CST and pancreastatin (PST)(26), another cleavage product of CgA. Our previous research showed that the intestinal barrier permeability is regulated by the antagonistic role of CST and PST, where CST reduced while PST increased permeability(6). We found that the increased length of the microvilli and the increased thickness of the mucus layer in the colon of CST-KO mice were fully reversible by bone-marrow transfer from WT mice. Combined with our previous findings that macrophages produce CST themselves and that bone-marrow transfer from WT mice into CST-KO mice results in near-normal levels of CST in the plasma(6), this suggests that the macrophages (and possibly other bone-marrow-derived cells) are major producers of CST in the colon. In line with our hypothesis that CST produced by infiltrating macrophages reduces mucus production, we found a reduced thickness of the mucus layer in inflamed regions of the colon of CD patients, which have a high density of infiltrated macrophages and other immune cells(27,28). Further supporting this hypothesis, intraperitoneal injection of CST in CST-KO mice

could reverse the expression of *MUC2*. Thus, based on the findings from this study, we expect that the elevated levels of CST produced by immune cells regulate the production of mucus in inflamed colon regions of CD patients.

An open question is what drives the cleavage of CgA to produce CST. Candidates are cathepsin L, an endolysosomal protease expressed by endocrine cells that co-localizes with CgA in chromaffin granules(29), and plasmin, the main enzyme of the fibrinolytic cascade(30). The expression of cathepsin L is increased in macrophages during mucosal inflammation of IBD patients(31), as well as in the colon of CST-KO mice(6). For plasmin, it has been shown that its inhibition prevents disease progression in acute colitis mouse models(32). However, its expression is downregulated in CST-KO mice(6). Finally, it seems possible that CgA is not only converted to CST by a host protease, but also by proteases produced by the gut bacteria(33), which would mean that the micro-environment in the gut defines the balance between CgA and CST.

Overall, our finding that CST regulates the mucus layer in the gut strengthens the emerging concept(6,31,32) that CST could be a potential new biomarker and/or therapeutic target for treatment of IBD and other gastrointestinal diseases.

Conclusion

CST produced by bone-marrow derived immune cells reduces production of the mucus layer in the intestine. Compared to healthy controls, IBD patients have increased stool levels of CST. This might contribute to the reduced mucus layer in inflamed colon regions of IBD patients. CST feces levels might be a therapeutic target or biomarker for IBD.

Materials and Methods

Mice

Male WT, CgA-KO and CST-KO mice (3 months old) on C57BL/6J background were used to study the intestine. All procedures relating to the WT and KO mice were approved by the Institutional Animal Care and Use Committee (UCSD) and all procedures and housing and handling of the animals were in accordance with National Institutes of Health animal care guidelines. CST-KO have been described before(19). CST-KO mice display hypertension, and a hyperadrenergic and insulin-resistant phenotype. Bone-marrow transplant from WT to CST-KO mice was performed as described previously(18).

Human colon biopsies and feces collection

Colon biopsies and feces samples were collected from patients with CD (N=39) and UC (N=36) (Table S1) in a cohort of long-standing CD and UC patients stratified for inflammation by colonoscopy. A flare-up or inflammation was defined as histological and endoscopic disease activity, whereas remission entailed absence of endoscopic inflammation and of histological disease activity. Non-inflamed biopsies were taken of the same colon segment > 2 cm from endoscopically visible disease. Feces from healthy controls (N=10) was also collected (average age 44 with range 38-54 years, 7/10 female) (Table S1). Informed consent was obtained and the study was conducted in accordance with the Institutional Board Review of the University Medical Center Utrecht (approval number NL 35053.041.11 & 11-050 for the IBD cohort and NL40331.078 for the healthy controls). Samples were collected in compliance with the Declaration of Helsinki.

CgA, CST and calprotectin levels in human feces

Feces was used to determine CgA and CST levels using ELISA (CUSABIO CSB-E17355h, CSB-E09153h) and calprotectin (HycultBiotech HK379).

Electron microscopy mouse colon

Human colon was fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate (pH 7.4) buffer overnight at 4°C. After washing in buffer tissue was postfixed for 1 h at RT in 1% osmium tetroxide and 1,5% potassium ferrocyanide in 0.1 M cacodylate buffer, washed in MQ, followed by one hour staining in *en bloc* with 2% uranyl acetate on ice. After washing in MQ, tissue was dehydrated in an ascending series of aqueous ethanol solutions and subsequently transferred via a mixture of aceton and Durcupan to pure Durcupan (Sigma) as embedding medium. Ultrathin sections (80 nm) were cut on a Leica Artros ultramicrotome, picked up on grids, stained with 2% uranyl acetate and lead citrate, air dried and examined in a JEOL JEM1400 electron microscope operating at 80 kV.

Electron microscopy human colon

Human colon was fixed with 2% glutaraldehyde in 0.1 M cacodylate (pH 7.4) buffer overnight at 4°C. Tissue was postfixed for 1 h at RT in 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer, washed in MQ, followed by one hour staining in en bloc with 2% uranyl acetate on ice. After washing in MQ, dehydrated in an ascending series of aqueous ethanol solutions and subsequently transferred via a mixture of aceton and Durcupan to pure Durcupan (Sigma) as embedding medium. Ultrathin sections (80 nm) were cut on a Leica ultramicrotome, and picked up on grids, stained with 2% uranyl acetate and lead citrate, air dried and examined in a JEOL JEM1400 electron microscope (JEOL) operating at 80 kV.

Processing and histology human & mouse colon

Human colon was fixed in carnoy-methanol to preserve the mucus layer. Mouse colon was fixed with 2.5% glutaraldehyde in 0.15 M cacodylate buffer. Mouse and human colon was dehydrated in ethanol series, xylene and paraffin. Afterwards, the tissue was carefully embedded in paraffin (formalin fixed and paraffin embedded (FFPE)) and sections of 5 µM were cut. Hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) staining were performed using standard techniques. Slides were mounted on silane coated glass slides (MUTO PURE CHEMICALS 511618) using Quick D mounting medium and imaged using the PerkinElmer Vectra (Vectra 3.0.3; PerkinElmer, MA) at 20x magnification.

Analysis Electron microscopy goblet cells and mucus layer in colon

The imageJ straight line tool was used to analyze the microvilli (from border till visible end in lumen) and mucus thickness (measured on top of microvilli). Additionally, the freehand drawing tool was used for measuring the goblet cell area.

Statistical data analysis

Data are expressed as mean ± SEM. D'Agostino & Pearson omnibus normality test was performed followed by one-way ANOVA with Bonferroni post-hoc tests or the Kruskal-Wallis with Dunn's. Significance is displayed in the graphs and a value of $p < 0.05$ was considered statistically significant.

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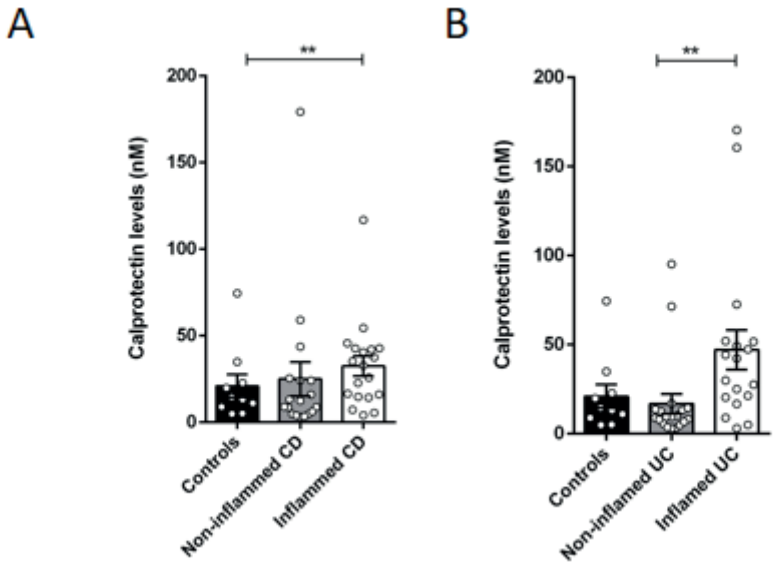


Fig. S1. Calprotectin levels correlates with IBD activity. (A) Calprotectin levels detected in feces of healthy donors (black) and Crohn's disease (CD) patients in remission (grey) or flare-up (white). (B) Same as panel A, but now for calprotectin levels in feces of ulcerative colitis (UC) patients. Kruskal-Wallis *: P<0.05; **: P<0.01; ***P<0.001.

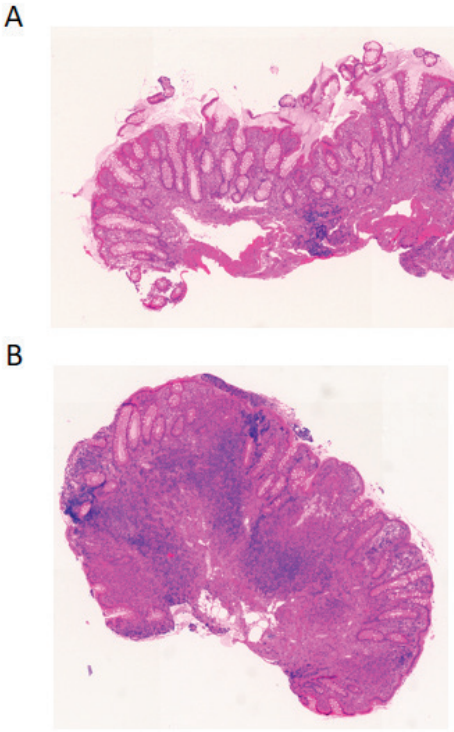


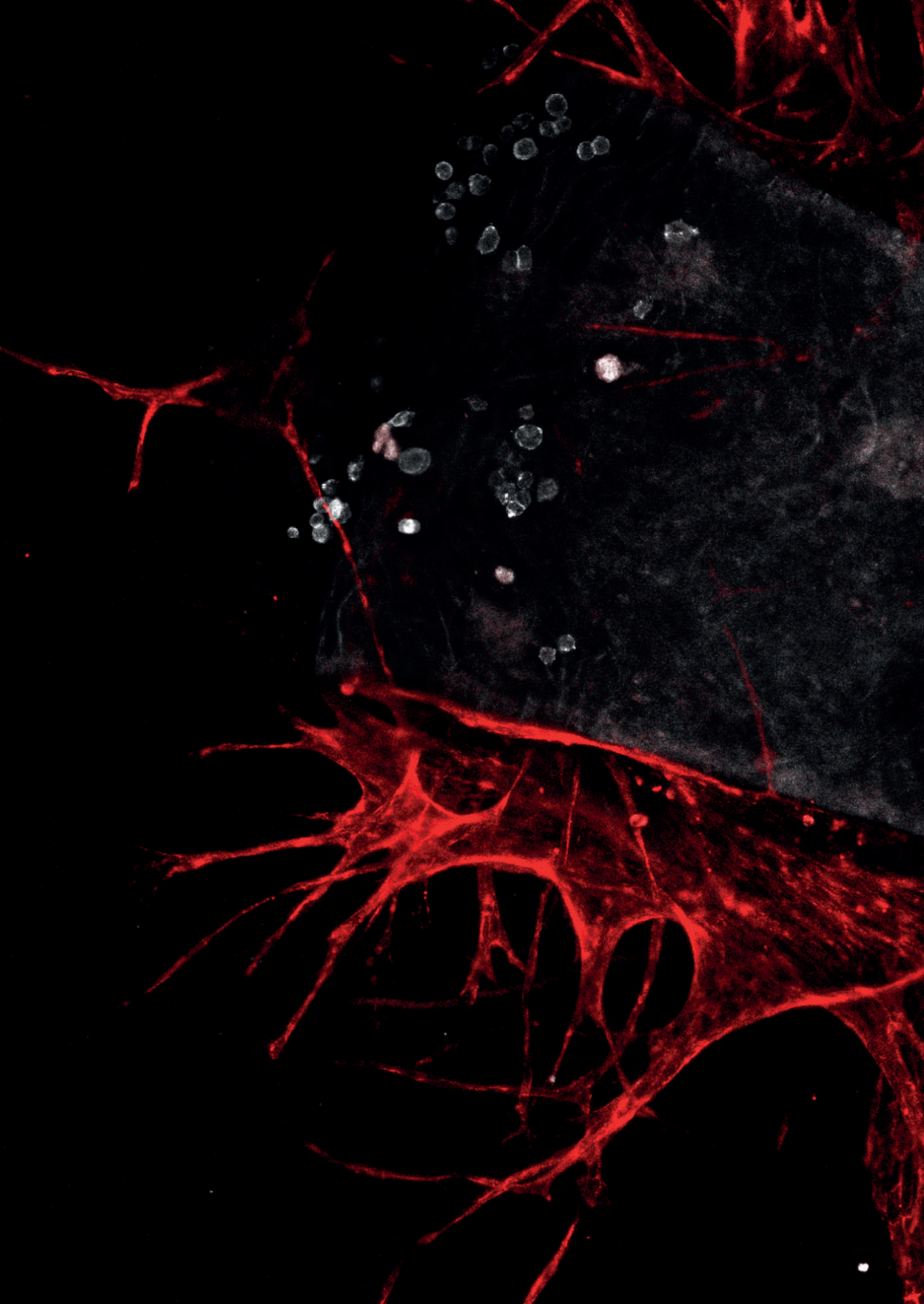
Fig. S2. HE stain colon Crohn's disease patient. Representative images of hematoxylin and eosin (HE) stained colon cross-sections of non-inflamed (A) and inflamed (B) colon tissue of Crohn's disease (CD) patients. Note the increased immune cell infiltration (blue cells) in the inflamed colon region.

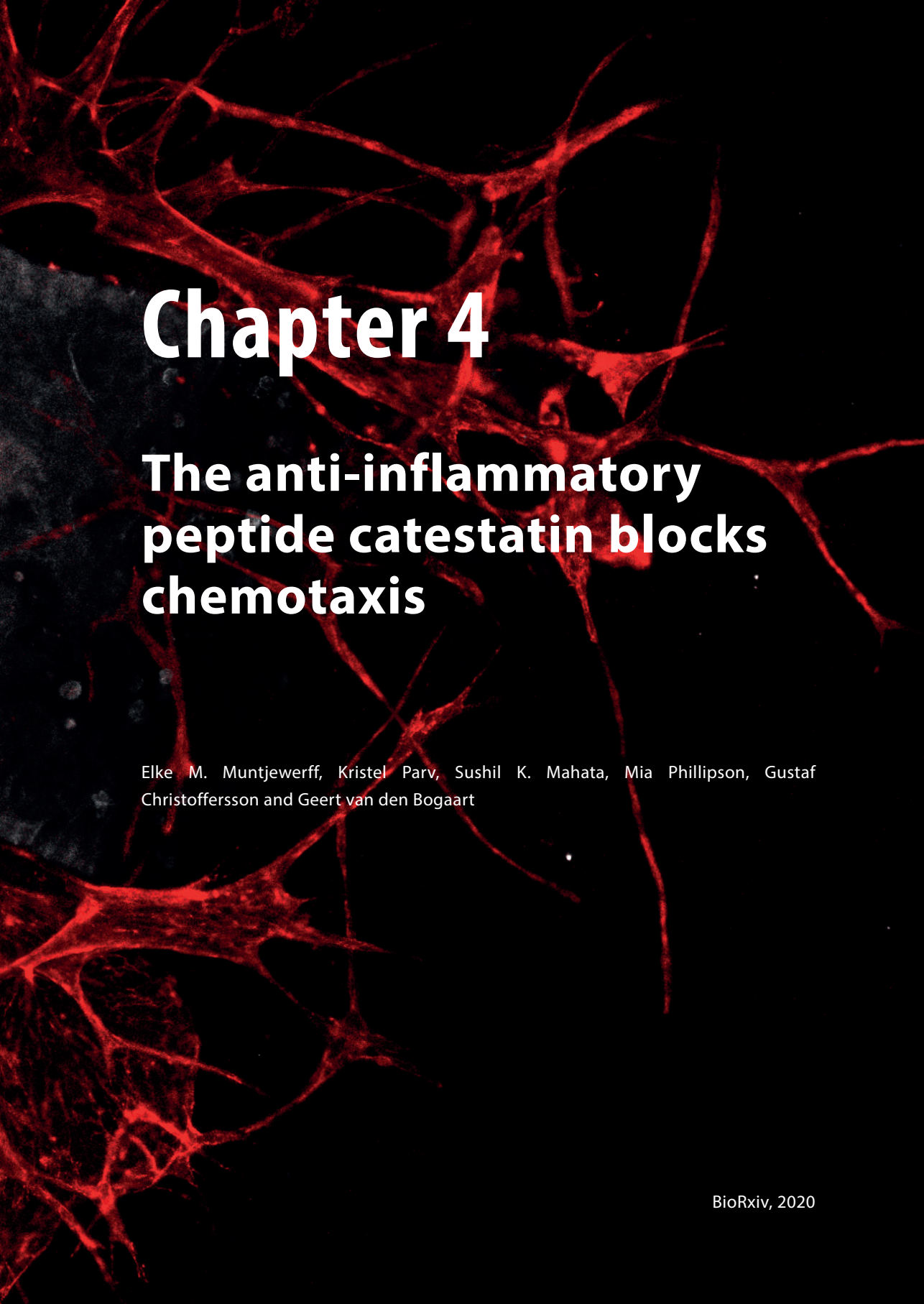
	Healthy controls (n=10)	**CD remission** (n=18)	**CD flare** (n=19)	**UC remission** (n=20)	**UC flare** (n=20)
Female (n, %)	7 (70%)	18 (67%)	10 (52%)	13 (65%)	9 (45%)
Age (average, range)	44 (38-54)	50 (22-71)	45 (22-72)	45 (20-66)	48 (24-75)
Disease duration (average, range)	-	23 (9-51)	18.5 (9-48)	16.5 (6-29)	19 (3-38)
Montreal (n, %)	-	L1 1 (5.5%)	0 (0%)	E1 3 (15%)	1 (5%)
		L2 7 (39%)	7 (37%)	E2 7 (35%)	5 (25%)
		L3 9 (50%)	11 (58%)	E3 10 (50%)	14 (70%)
		L2 + L4 0 (0%)	1 (5%)		
		L3 + L4 1 (5.5%)	0 (0%)		
IBD medication (%) *None*	50 (100%)	3 (17%)	5 (26%)	3 (15%)	0 (0%)
Mesalazine preparations		2 (11%)	2 (10.5%)	12 (60%)	17 (85%)
Corticosteroids		0 (0%)	1 (5%)	0 (0%)	5 (25%)
Immunosuppressives		12 (67%)	4 (21%)	8 (40%)	5 (25%)
Biologicals		3 (17%)	9 (47%)	1 (5%)	0 (0%)

Table 1. Patient characteristics for feces measurements. Characteristics of the patients at time of inclusion for stool measurements. As an indication for the extent of disease, the location has been scored according to the Montreal classification. For CD; L1 is ileal disease, L2 is colonic disease, L3 is ileocolonic disease, and L4 is isolated upper gastro-intestinal tract. For UC; E1 is distal to the rectosigmoid junction (proctitis), E2 is distal to the splenic flexure (left-sided) and E3 is proximal to the splenic flexure (extensive). Medication entails therapeutic categories in use for IBD; other therapies are not included. Some patients use more then one drug which all have been scored separately. Biologicals comprised only anti-TNF-α compounds. CD: Crohn's disease, IBD: inflammatory bowel disease, UC: ulcerative colitis.

	Disease	**Sex**	**Age**	**Montreal**	**Colon segment**	**Pathology**
1	Healthy control	M	63	-	Ascendens	-
2	Ulcerative colitis	M	27	S3E3	Transversum	Severe active inflammation
3	Crohn's disease	M	61	A2L1B1	Ascendens/ cecum	Mild chronic inflammation
4	Crohn's disease	M	52	A1L2B1+p	Sigmoid	a: No inflammation b: Chronically active inflammation

Table 2. Patient characteristics for electron microscopy. Characteristics of the patient at time of inclusion for electron microscopy on colonic biopsies. Indication for colonoscopy for the healthy control was a positive iFOBT. For patients paired biopsies ware taken, a and b.





Chapter 4

The anti-inflammatory peptide catestatin blocks chemotaxis

Elke M. Muntjewerff, Kristel Parv, Sushil K. Mahata, Mia Phillipson, Gustaf Christoffersson and Geert van den Bogaart

BioRxiv, 2020

Abstract

Increased levels of the anti-inflammatory peptide catestatin (CST), a cleavage product of the pro-hormone chromogranin A, correlates with less severe outcomes in hypertension, colitis and diabetes. However, it is unknown how CST reduces the infiltration of monocytes and macrophages in inflamed tissues. Here, we report that CST blocks leukocyte migration towards inflammatory chemokines. By *in vitro* and *in vivo* migration assays, we show that although CST itself is weakly chemotactic, it blocks migration of monocytes and granulocytes to inflammatory attracting factor CC-chemokine ligand 2 (CCL2) and macrophage inflammatory protein 2 (MIP-2). Moreover, it directs CX_3CR1^+ macrophages away from pancreatic islets. These findings support the emerging notion that CST is a key anti-inflammatory modulator.

Introduction

As an immunological response to inflammation, monocytes, granulocytes and leukocytes are attracted to inflamed tissues by chemokines such as CC-chemokine ligand 2 (CCL2, a.k.a. MCP-1) and macrophage inflammatory protein 2 (MIP-2, a.k.a. CXCL2) (1). However, to avoid an excessive response, leukocyte infiltration should be halted for resolution of inflammation, but the mechanisms that govern this are unknown (2). Here, we addressed the potential chemotactic effect of chromogranin A (CgA)-derived peptide Catestatin (CST: $hCgA_{352-372}$) (3). While CST circulates at low nM range, the local concentrations were detected in the µM range in mouse tissues (3–6).

Being an anti-inflammatory peptide, CST reduces inflammation in cardiac and chronic inflammatory diseases (3,7–9). Despite the chemotactic effects of CST (7,10,11), administration of exogenous CST reduces monocyte and macrophage infiltration in the liver, heart and gut in mouse models of type II diabetes, hypertension, atherosclerosis and colitis (4,7,8,12,13). In a colitis model, CST also reduced granulocyte infiltration in the colon (8). In line with this, the adrenal gland, heart, and gut of CST knockout mice display increased macrophage infiltration (4,7,12). In this study, we show that while CST itself is weakly chemotactic, it blocks the extravasation and migration of phagocytes both *in vitro* and *in vivo*. Thus, the anti-inflammatory effects of CST are partly the result of redirecting monocytes and granulocytes away from the inflammation sites.

Results & Discussion

Although human blood monocytes migrated towards a high (but physiological) concentration of CST (5 µM), this was less efficient compared to the canonical inflammatory chemokine CCL2 (0.5 nM) (Fig. 1A-C), reinforcing a weak chemoattractive effect of CST (7,10,11). To confirm this *in vivo*, we performed imaging of the cremaster muscle (Fig. 1D) (14). Upon perfusion of the muscle with CST (5 µM), phagocytes (monocytes and granulocytes) decreased their speed and attached to the vessel wall with similar efficiency as of the inflammatory chemotactic agent MIP-2 (0.5 nM) (Fig. 1D-F, Fig. S1). Thus, both our *in vivo* and *in vitro* migration assays show that CST is weakly chemotactic, raising the question how CST can reduce monocyte and granulocyte infiltration in inflamed tissues such as the liver (diet induced obese mice), intestine (colitis model), heart (hypertension model) and atheromatous plaques (atherosclerosis model) (4,7,8,12,13).

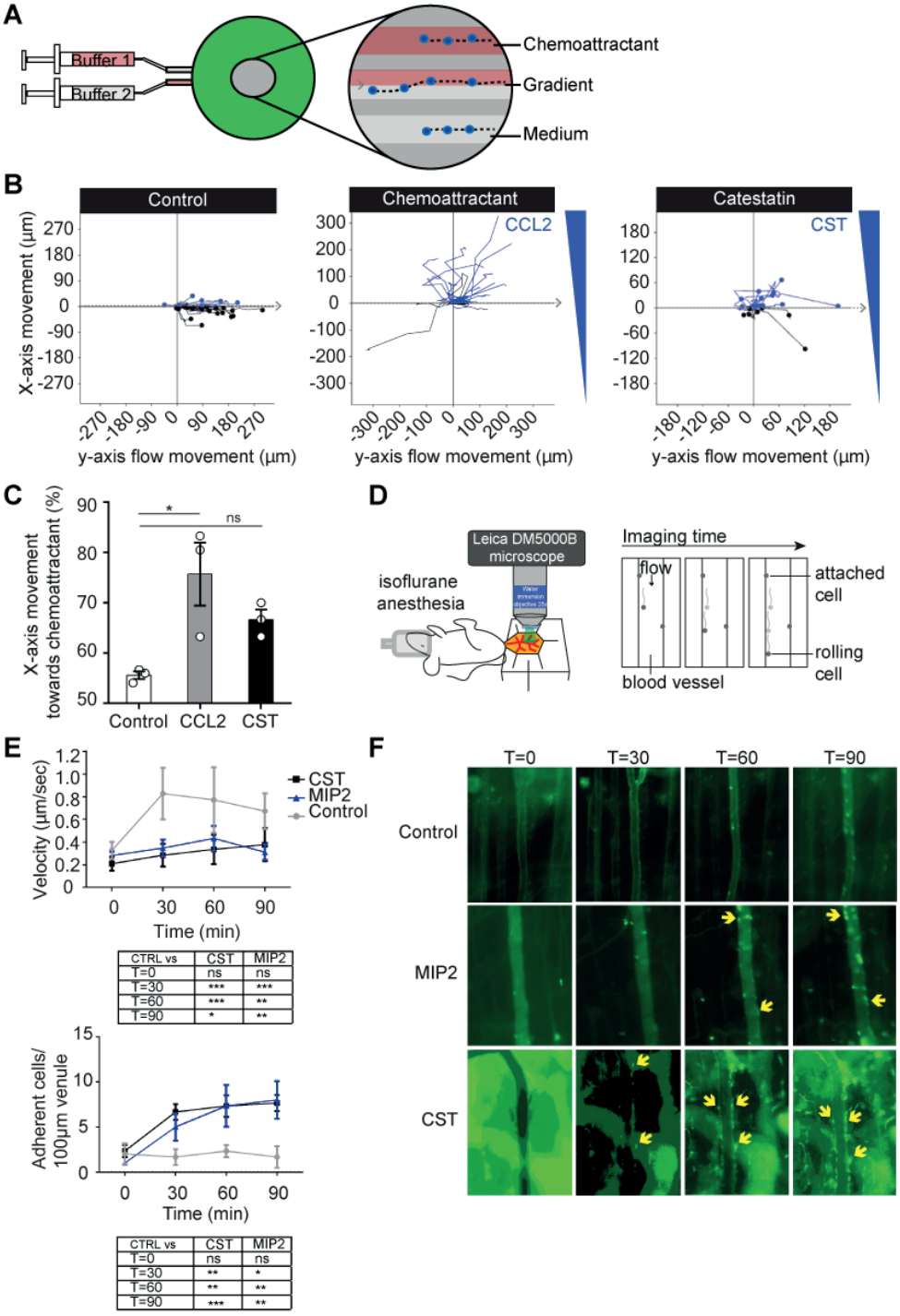

A
Buffer 1
Buffer 2
Chemoattractant
Gradient
Medium
B
Control
Chemoattractant
Catestatin
CCL2
CST
X-axis movement (µm)
y-axis flow movement (µm)
C
X-axis movement towards chemoattractant (%)
Control
CCL2
CST
ns
D
Leica DM5000B microscope
isoflurane anesthesia
Imaging time
flow
attached cell
rolling cell
blood vessel
E
Velocity (µm/sec)
Time (min)
CST
MIP2
Control
CTRL vs | CST | MIP2
T=0 | ns | ns
T=30 | *** | ***
T=60 | *** | **
T=90 | * | **
Adherent cells/ 100µm venule
Time (min)
CTRL vs | CST | MIP2
T=0 | ns | ns
T=30 | ** | *
T=60 | ** | **
T=90 | *** | **
F
T=0
T=30
T=60
T=90
Control
MIP2
CST

Fig. 1: CST is weakly chemotactic. (A) Scheme showing set-up of Gradientech migration assay. Two syringes filled with buffer +/- chemoattractant were connected to the device (green) to create a flow (*x*-direction) and perpendicular (*y*) cytokine gradient. The inset shows migration of monocytes along the flow and towards the chemoattractant. **(B)** Representative tracks of human monocytes showing the *x*- and *y*-movement of individual cells upon exposure to the indicated buffer, 5 µM CST or 0.5 nM CCL2. **(C)** Quantification of panel B (N=3). **(D)** Scheme showing set-up of cremaster muscle imaging in mice to visualize phagocyte (monocytes and granulocytes) extravasation *in vivo*. **(E)** Phagocyte rolling velocity (top) and attachment (bottom) upon overflowing the muscle with buffer (control, gray), 0.5 nM MIP-2 (blue) or 5 µM CST (black) (N=3, two-way ANOVA). **(F)** Representative images of granulocyte attachment as visualized by Ly6G-mAb (green) to the vessel wall upon only buffer, MIP-2 or CST stimulation. *: P<0.05; **: P<0.01; ***P<0.001; ns: not significant.

To address how CST affects macrophage chemotaxis to inflamed tissues, we used the aortic ring vessel model (15) (Fig. 2A), which is based on the co-embedding of part of the aorta of *Cx$_3$cr1$^{+/gfp}$*transgenic mice adjacent to isolated pancreatic islets (16). These islets secrete chemokines, such as vascular endothelial growth factor (VEGF)-A, resulting in the directional macrophage migration from the aortic ring as well as vessel growth towards the pancreatic islets. Migration of CX$_3$CR1$^+$macrophages from the aortic ring was visualized by fluorescence microscopy (17) (Fig. 2B, S2). As expected, the CX$_3$CR1-macrophages moved towards the pancreatic islets in absence of CST (Fig 2B). However, perfusing the aortic ring with CST (5 µM) resulted in a lower number of CX$_3$CR1^{+GFP} macrophages migrating towards the pancreatic islets (Fig 2B), indicating that CST blocked directional migration. Interestingly, we also observed that CST is pro-angiogenic, as it increased both the amount and length of the sprouts and branches emanating from the aortic rings (Fig. 2C-D, S3).

The loss of directional cell migration to the pancreatic islets might be caused by blockage of chemokine-induced cell migration by CST. To investigate this possibility, we performed intravital imaging of the cremaster muscle, but this time for CST in combination with MIP-2. This resulted in the inverse effect compared to CST or MIP-2 alone: release of attached cells from the vessel wall and reduced migration of cells into the tissue (Fig. 2E, S4), indicating that despite being weakly chemotactic, CST blocks MIP-2 elicited phagocyte recruitment. To further confirm this, we performed an *in vitro* migration assay, where human monocytes were stimulated with a gradient of CCL2 in presence of CST (Fig. 2F). Similar to our findings with the intravital imaging, CST blocked monocyte migration towards the CCL2.

Although CST counteracts the chemoattraction by inflammatory cytokines (Fig. 2G), the question remains open which receptor(s) CST utilize to exert these effects on cell migration. We speculate that this might be a G-protein coupled receptor (GPCR),

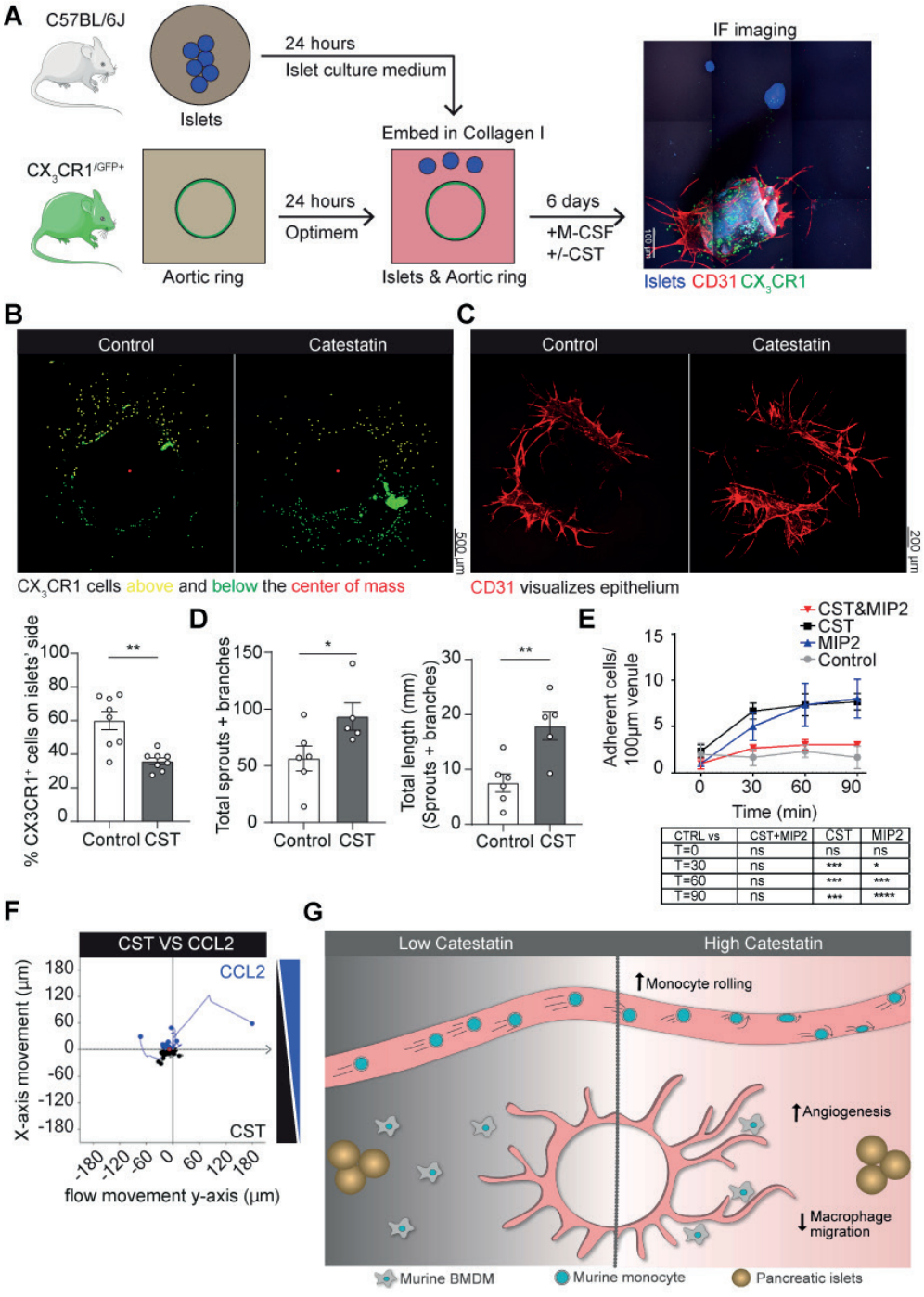

A
C57BL/6J
Islets
24 hours
Islet culture medium
Embed in Collagen I
CX3CR1/GFP+
Aortic ring
24 hours
Optimem
Islets & Aortic ring
6 days
+M-CSF
+/-CST
IF imaging
Islets CD31 CX3CR1
B
Control
Catestatin
500 µm
CX3CR1 cells above and below the center of mass
C
Control
Catestatin
200 µm
CD31 visualizes epithelium
D
% CX3CR1+ cells on islets' side
Control CST
Total sprouts + branches
Control CST
Total length (mm) (Sprouts + branches)
Control CST
E
CST&MIP2
CST
MIP2
Control
Adherent cells/ 100µm venule
Time (min)
CTRL vs | CST+MIP2 | CST | MIP2
T=0 | ns | ns | ns
T=30 | ns | *** | *
T=60 | ns | *** | ***
T=90 | ns | *** | ****
F
CST VS CCL2
CCL2
CST
X-axis movement (µm)
flow movement y-axis (µm)
G
Low Catestatin
High Catestatin
Monocyte rolling
Angiogenesis
Macrophage migration
Murine BMDM
Murine monocyte
Pancreatic islets

Fig. 2: CST blocks migration induced by inflammatory chemokines and promotes angiogenesis. (A) Scheme showing set-up of aortic ring assay. Aortic ring was isolated from CX_3CR1-GFP mice and embedded adjacent to pancreatic islets in collagen I. Image shows islets (blue), CD31 (red) and CX_3CR1 (green). **(B)** Representative images of CX3CR1-macrophage migration upon control or CST stimulation of the aortic ring. The graph shows the percentage of cells above (yellow) the center of mass (N=8). **(C)** Representative images of vessels by CD31 (red) upon control or CST stimulation of the aortic ring. **(D)** Quantification of angiogenesis. Total number of sprouts and branches (left) and their length (right) (N=5-6). **(E)** Cremaster muscle imaging. Phagocyte attachment to vessel wall upon overflowing the muscle with buffer (control, gray) and buffer with the chemoattractant MIP-2 (blue), CST (black) or both (red) (N=3, two-way ANOVA). **(F)** Gradientech migration assay. Representative *x*- and *y*-movement of human monocytes exposed to opposite gradients of CST and CCL2 (N=3). **(G)** Model showing leukocyte extravasation in presence of low and high concentrations of CST. Mann-Whitney test *: $P<0.05$; **: $P<0.01$; ***$P<0.001$; ****$P<0.0001$; ns: not significant.

since GPCRs are actively involved in leukocyte migration (18) coupled with expression of GPCRs in all cell types responsive to catestatin (e.g. monocytes (10), neutrophils (19,20), macrophages (4,7,8,12,13), endothelial (13,21) and mast cells (11)), we speculate that CST might act through this receptor type. We have not only shown how CST reduces the infiltration of monocytes and macrophages in inflamed tissues (4,7,8,12,13), but offer a possible mechanistic explanation for the correlation of CST levels with improved disease outcome in patients suffering from chronic diseases (4–6), reinforcing CST as a therapeutic target for treatment of diseases associated with chronic inflammation.

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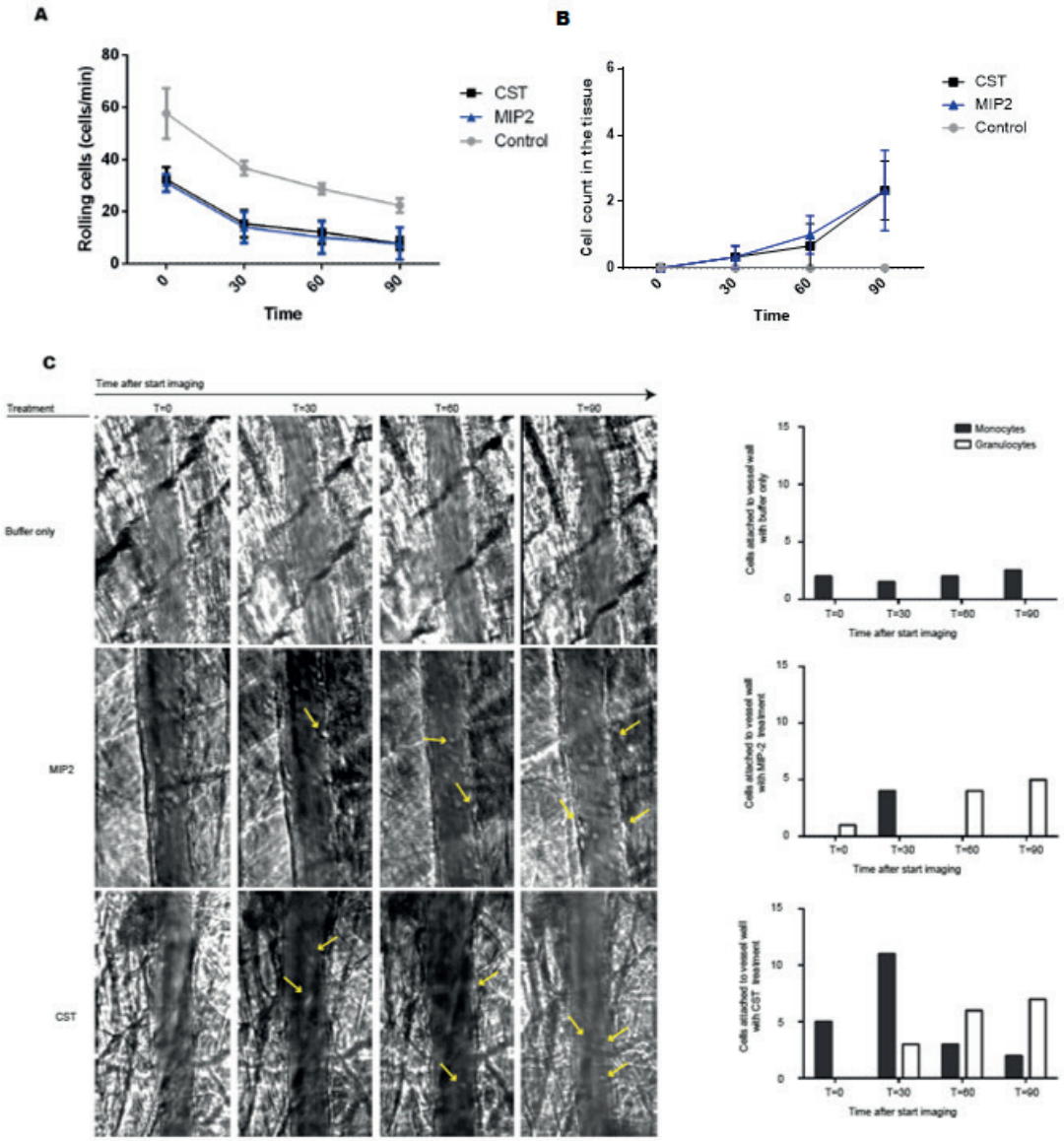
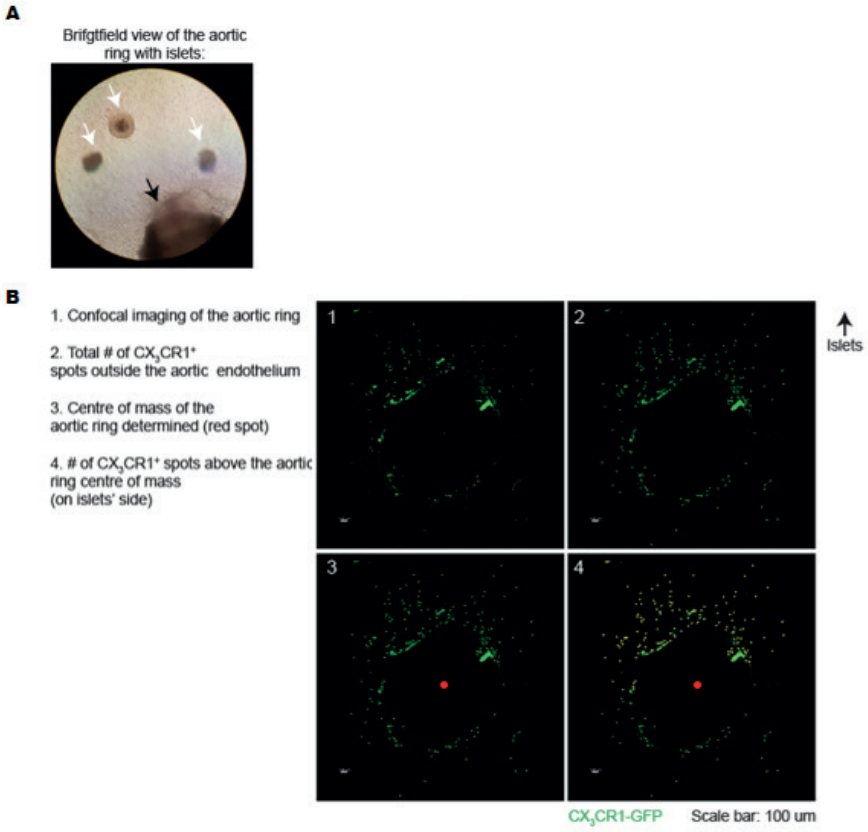
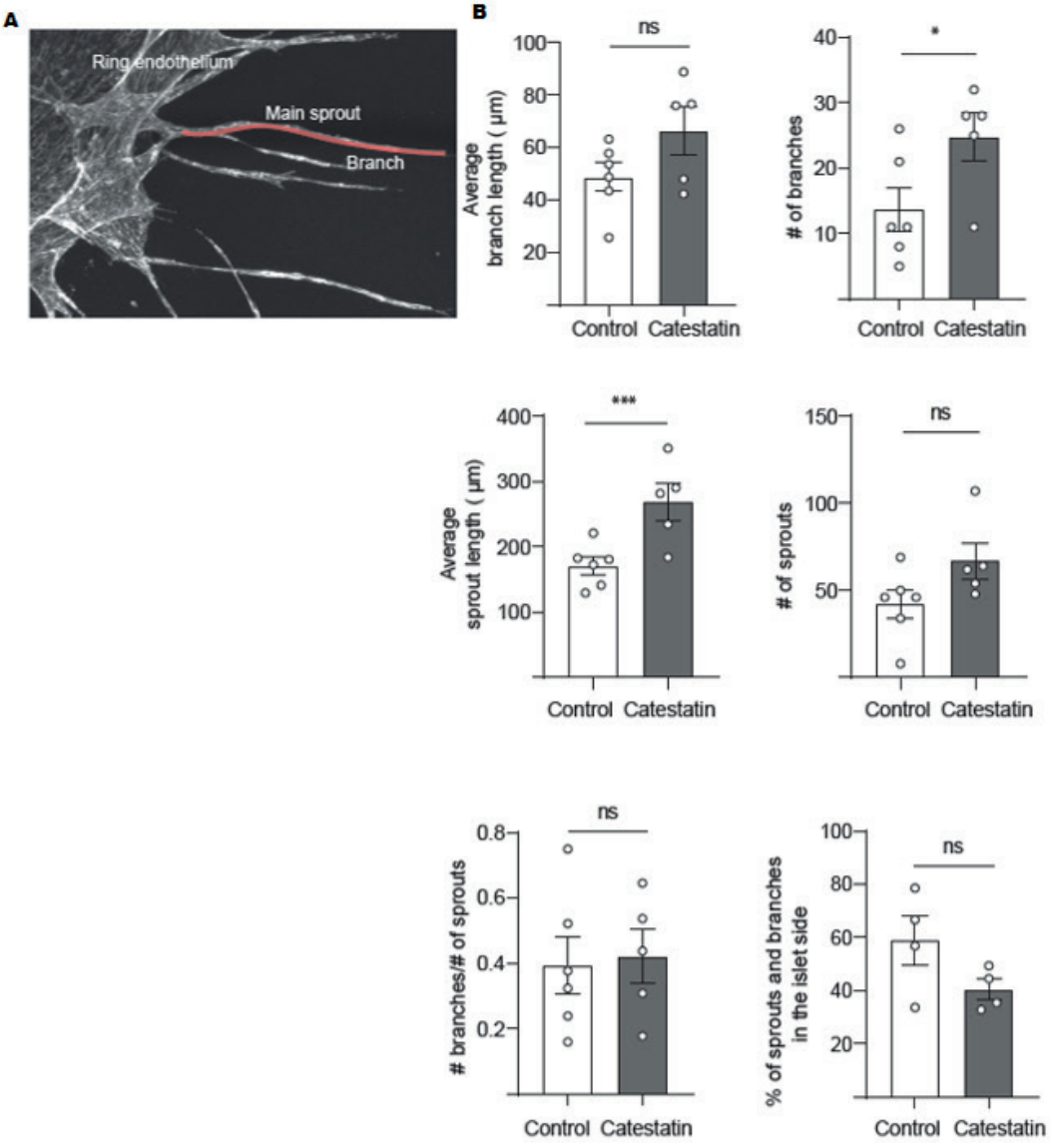


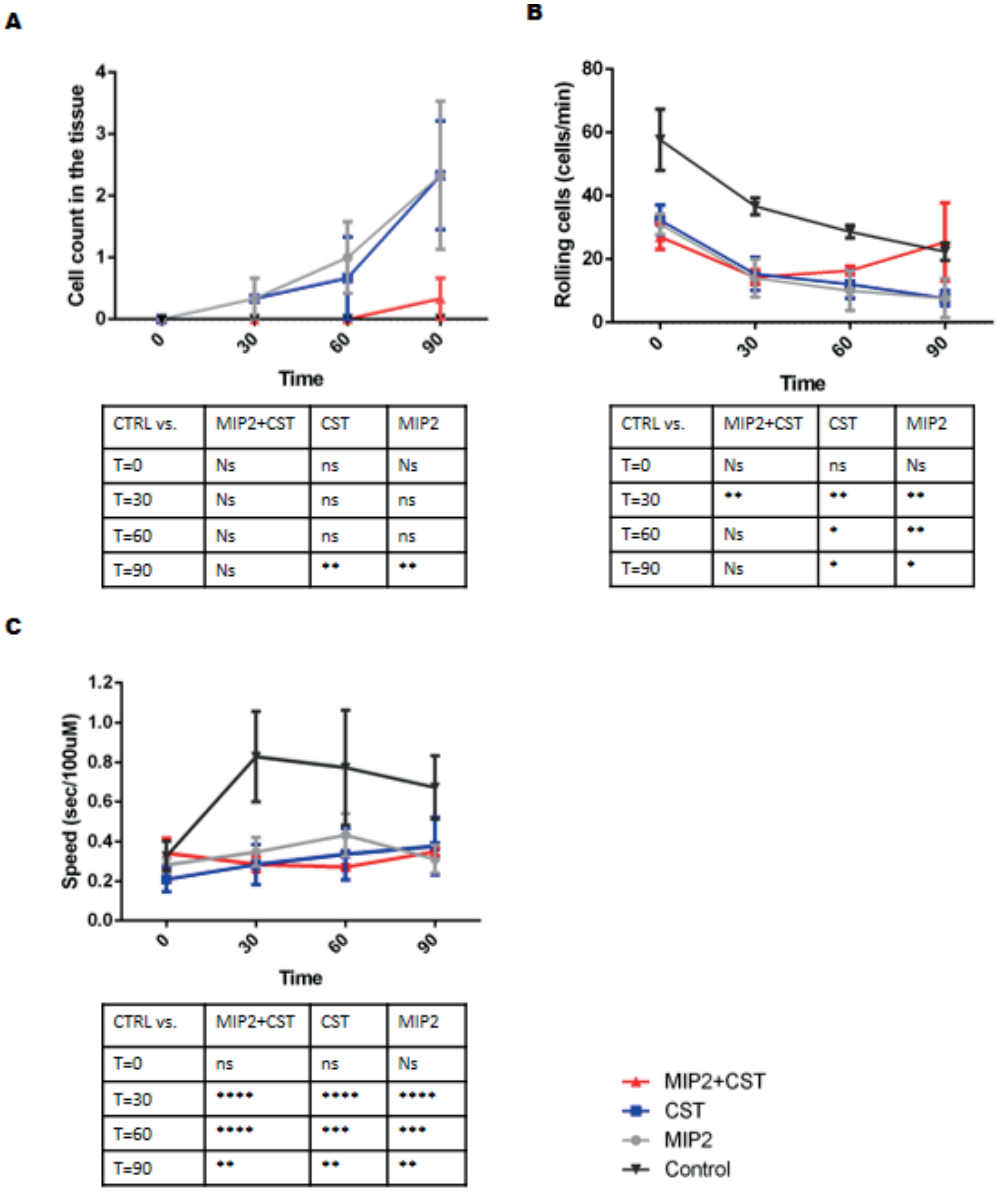
Sup. 1: Attachment of granulocytes and monocytes to vessel wall. (**A**) Venules of the cremaster muscle were overflown with bicarbonate-buffered saline buffer (buffer only control), the chemoattractant MIP-2 or CST as shown in main Fig. 1D-F. Graph shows quantification of rolling cells (cells/min). (**B**) Quantification of cell in tissue. (**C**) Representative brightfield snapshots of *in vivo* cremaster muscle imaging as in main figure 1D-F. (**C**) Quantification of adherent granulocytes (visualized by Ly6G-mAb, main Fig. 1F) and monocytes (brightfield, panel C) after 0, 30, 60 and 90 minutes (N=1-2).



Sup. 2: Quantification of CX3XR1+ cell movement in the aortic ring model. (**A**) Brightfield image of the aortic ring with islets. (**B**) Description of CX3CR1-cell movement quantification by determination of total amount outside the aortic ring (endothelium), center of mass (red spot) and the islet side (black arrow).



Sup. 3: Branches and sprouts in the aortic ring assay. (**A**) Representative images of angiogenesis quantification of main figure 2C-D. The images show the ring endothelium, main sprout (red), branch (gray) (**B**) Quantification of total number of sprouts and branches separately and their length (N=5-6). Mann-Whitney test *: P<0.05; ***P<0.001; ns: not significant.

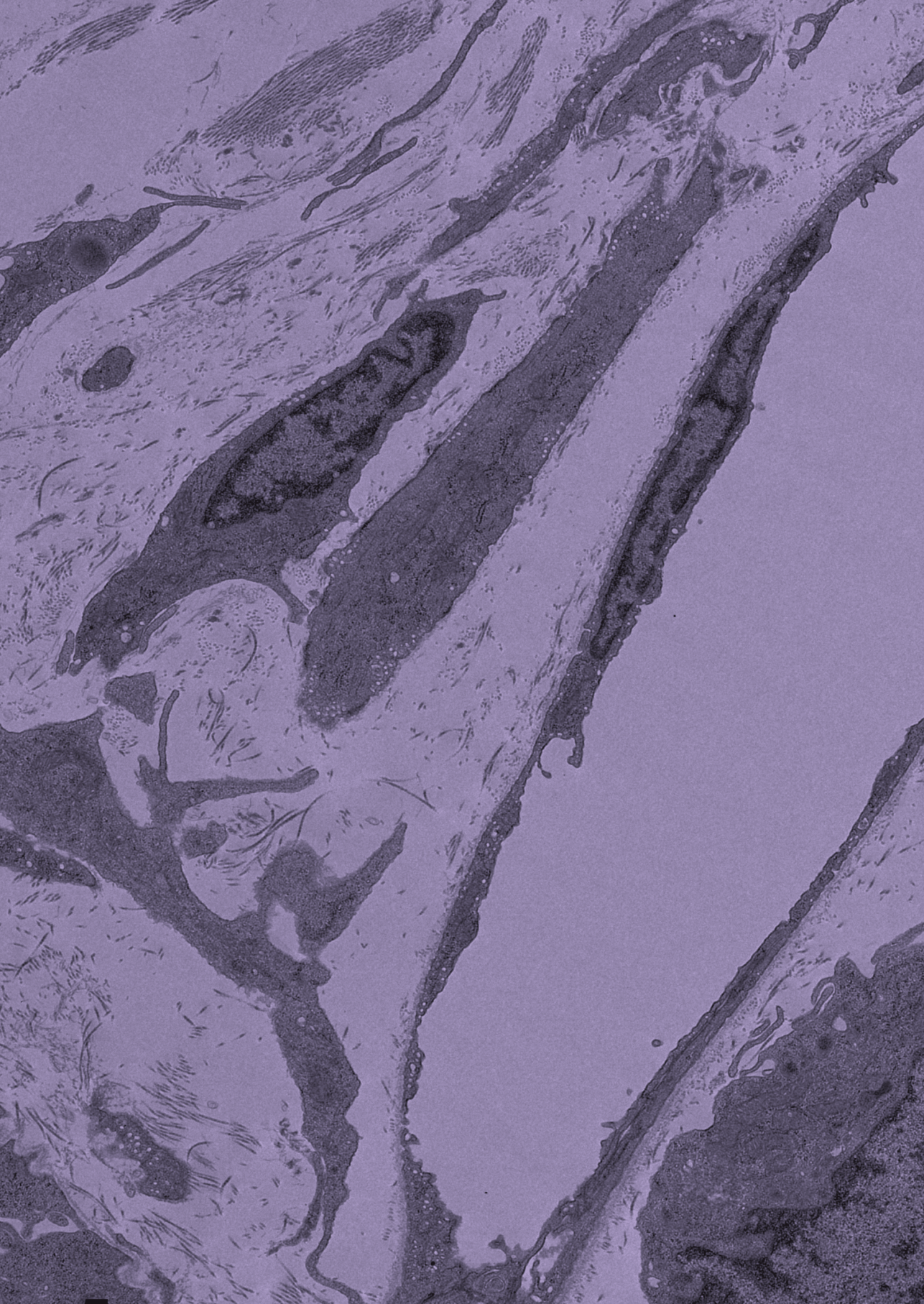


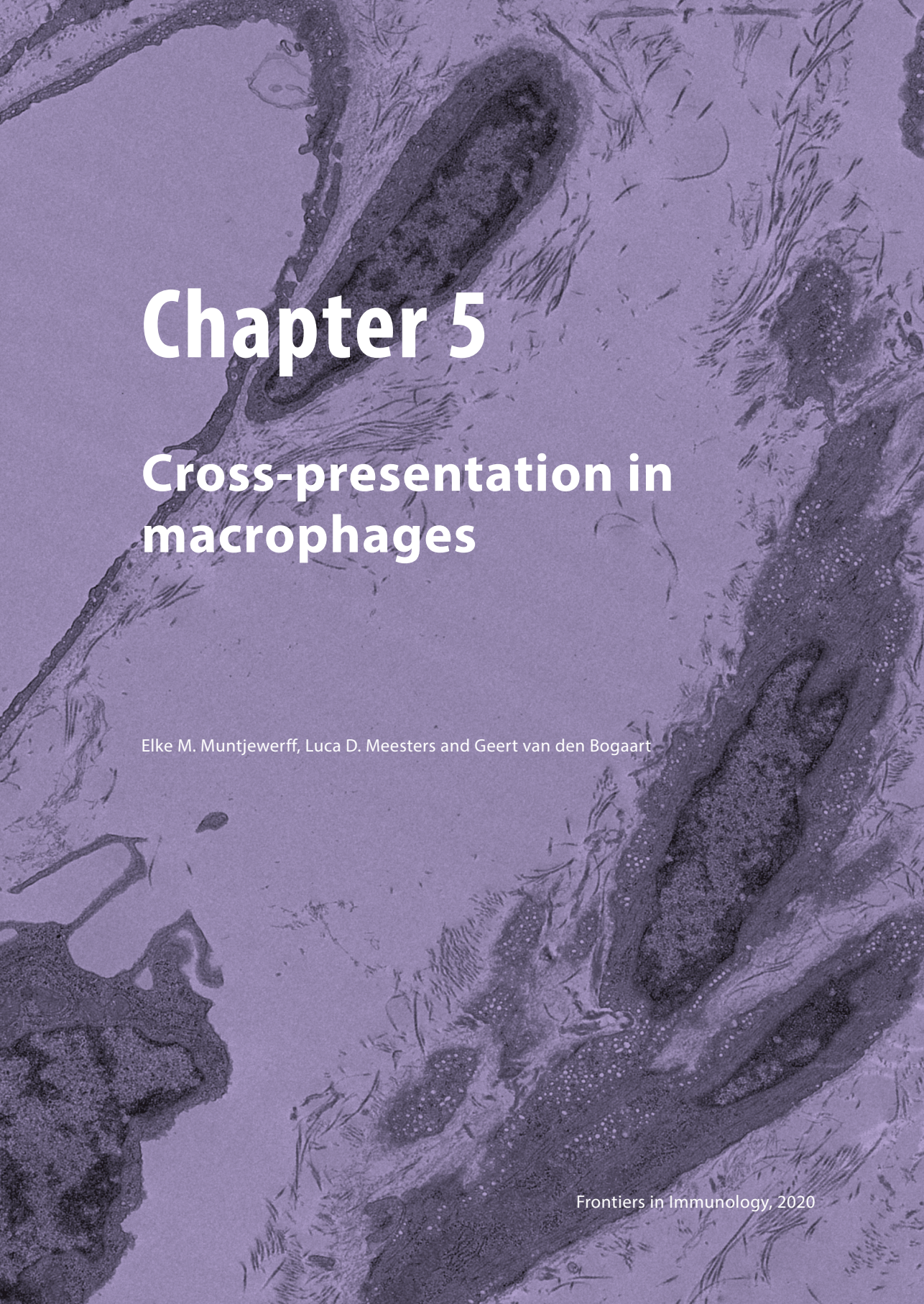
CTRL vs.	MIP2+CST	CST	MIP2
T=0	Ns	ns	Ns
T=30	Ns	ns	ns
T=60	Ns	ns	ns
T=90	Ns	**	**

CTRL vs.	MIP2+CST	CST	MIP2
T=0	Ns	ns	Ns
T=30	**	**	**
T=60	Ns	*	**
T=90	Ns	*	*

CTRL vs.	MIP2+CST	CST	MIP2
T=0	ns	ns	Ns
T=30	****	****	****
T=60	****	***	***
T=90	**	**	**

Sup. 4: The combination of CST and MIP-2 reduced chemotaxis. Venules of the cremaster muscle were overflown with bicarbonate-buffered saline buffer (buffer only control), the chemoattractant MIP-2 or CST, as shown in main Fig. 1D. Graph shows quantification of tissue migration (**A**), rolling cells (cells/min) (**B**) and velocity (**C**) upon CST, MIP-2 or stimulation with both (N=3, two-way ANOVA) *: P<0.05; **: P<0.01; ***P<0.001; ****P<0.0001; ns: not significant.





Chapter 5

Cross-presentation in macrophages

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Abstract

The contribution of dendritic cell (DC) antigen cross-presentation to the activation of $CD8^+$ T lymphocytes for immune defense against tumors, viruses and intracellular pathogens has been recognized widely. Although originally thought to be an exclusive characteristic of DCs, recently also other immune cells, particularly macrophages, have been shown capable of cross-presentation. Here we provide an overview of *in vitro* and *in vivo* evidence on cross-presentation by macrophages. As we discuss, it is now firmly established that various types of tissue-resident macrophages are able to cross-present via similar cellular pathways as DCs. This is based on a wide range of antigens in macrophages from many different tissue origins such as blood, tumors and lymphoid tissue. However, the physiological relevance of macrophage cross-presentation with potential contributions to activation of $CD8^+$ T lymphocytes is still mostly unknown. While cross-presentation by various types of pro-inflammatory macrophages might be involved in cross-priming of naive $CD8^+$ T lymphocytes, it might also be involved in local re-activation of memory and/or effector $CD8^+$ T lymphocytes. Moreover, cross-presentation by anti-inflammatory macrophages could be related to immune tolerance. Since cross-presentation promotes the initiation and potentiation of antigen-specific $CD8^+$ T lymphocyte responses, stimulating macrophages to cross-present antigen might be a promising strategy for anti-tumor or anti-viral therapies.

Introduction

Since the first evidence of their antigen cross-presenting capacities for activation of naive $CD8^+$ T lymphocytes in 1976, dendritic cells (DC) have been heavily studied for the mechanisms and physiological roles of cross-presentation (1). Antigen cross-presentation is crucial for the initiation of adaptive immune responses against cancer, viruses and numerous other intracellular pathogens. During this process, antigen presenting cells (APCs) present peptides derived from ingested antigens in their major histocompatibility complex class I (MHC-I) protein complex to naive $CD8^+$ T lymphocytes. If the DCs also provide sufficient levels of co-stimulatory receptors (e.g., CD80, CD86) and cytokines (interleukin-12; IL-12), cross-presentation results in the activation of naive $CD8^+$ T lymphocytes to effector cytolytic T cells in a process called cross-priming. Effector cytolytic T cells can induce apoptosis in infected or malignant cells (2,3). Since DCs were assumed the only (or at least main) cross-presenting cells capable of cross-priming naive $CD8^+$ T lymphocytes, by far most research has been focused on cross-presentation pathways in DCs. However, recently also macrophages have been shown to be capable of antigen cross-presentation and this might have large implications for our understanding of $CD8^+$ T lymphocyte responses. Therefore, the aim of this review is to provide an overview of the current studies covering the roles and mechanisms of antigen cross-presentation by macrophages.

Role of dendritic cells and macrophages in $CD8^+$ T lymphocyte activation

During their lifetime, DCs can exist in two discrete stages: immature and mature. Immature DCs are overall considered to be better in endocytosis and protein processing, and their primary role is to sample antigen in the circulation and peripheral tissues. The recognition of antigen by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), results in the maturation of the DCs. The resulting mature DC can migrate to the lymphoid organs, where it can activate antigen-specific $CD8^+$ T lymphocytes by cross-presentation (4). DCs can be categorized into subsets based on their cross-priming ability and origin. Many studies are devoted to characterize the capacity of various DC subsets to cross-present to $CD8^+$ T lymphocytes in mouse and human by for example the use of RNA sequencing and lineage tracing, which has been extensively reviewed elsewhere (2,5,6). Briefly, the main subsets in human are: plasmacytoid $CD123^+CD303^+CD304^+$ DCs (pDC) and conventional DCs (cDC) including $CD1a^+CD11c^{high}XCR1^-BDCA\text{-}1^+$ (cDC2) and $CD1a^-CD11c^{low}$ $CLEC9A^+XCR1^+BDCA\text{-}3^+CD141^+$ (cDC1) (2,5,6). All these subsets have the capacity to cross-present, but due to the high expression of MHC-I pathway genes, cDC1s are considered as the most efficient cross-presenting cells in human (2,5,6). The same subsets are present in mice, but then based on expression of CD11c and MHC class II in combination with CD4,

CD8α, CD11b, XCR1, CLEC9a, CD103 and CD205. The mouse classical DCs express either CD11b (equivalent to human cDC2) or CD8α, XCR1, CLEC9A (also known as DNGR1) and CD103 (equivalent to human cDC1), where the cross-presenting efficiency depends on the expression of CD8 (in lymphoid tissue), making the classical $CD8^+$ DC the best mouse subset for activation of cytolytic T lymphocytes (2,5,6).

Tissue macrophages such as liver Kupffer cells, spleen red-pulp and large peritoneal macrophages develop during embryogenesis, where they originate from precursors in the yolk sac, fetal liver and bone marrow (extensively reviewed in (7)). These embryonically-derived macrophages become tissue-resident macrophages that can propagate via self-renewal (7,8). Later in life, the hematopoietic stem cells in the bone marrow give rise to $LY6C^+$ monocytes in mice or $CD14^+CD16^+$ monocytes in human, which can subsequently be recruited from the blood into the tissues, such as sites of infection or tumors, to either promote further inflammation or tissue repair (8–10). Upon arrival, these monocytes can differentiate into pro- or anti-inflammatory monocyte-derived macrophages, depending on the PRR signaling, growth factors and cytokines present in the tissue. The pro-inflammatory, also known as M1 or classically-activated, macrophages are activated by signaling from PRRs or inflammatory cytokines (e.g., interferon-γ; IFN-γ) and express pro-inflammatory cytokines, IL-6, TNF-α, IL-1β, and inducible nitric-oxide synthase (iNOS) (6,11). The anti-inflammatory, also called M2, pro-resolving or alternatively-activated, macrophages are stimulated by interleukin-4 (IL-4) or IL-13 and express arginase 1 (Arg1), the mannose receptor CD206 and the IL-4 receptor α-chain (IL-4Rα). The main function of the pro-inflammatory macrophages is to eliminate tumors and pathogens by production of inflammatory cytokines and phagocytosis, while the main functions of the anti-inflammatory macrophages are tissue repair and homeostasis (12–14).When there is space in the tissue niche, blood-recruited monocytes might also differentiate into tissue macrophages (8). Both embryonically-derived and monocyte-derived macrophages are able to sense and phagocytose tumor cells and pathogens (15). The precise phenotype and access to antigen of tissue-resident macrophages depends on the tissue, but especially spleen, lymph node, liver and peritoneal macrophages (Fig. 1) constantly encounter blood- or lymph-borne antigens, which means that they are ideally positioned for antigen uptake and they are thus in principle well-positioned for cross-presentation to $CD8^+$ T lymphocytes.

Although antigen cross-presentation by macrophages is less well understood than for DCs, it is increasingly clear that especially pro-inflammatory macrophages are capable of cross-presentation. The function of this cross-presentation is unclear, but evidence suggests it might be important for cross-priming of naive $CD8^+$ T lymphocytes similar as in DCs. In addition, it might aid in the activation of memory $CD8^+$ T lymphocytes,

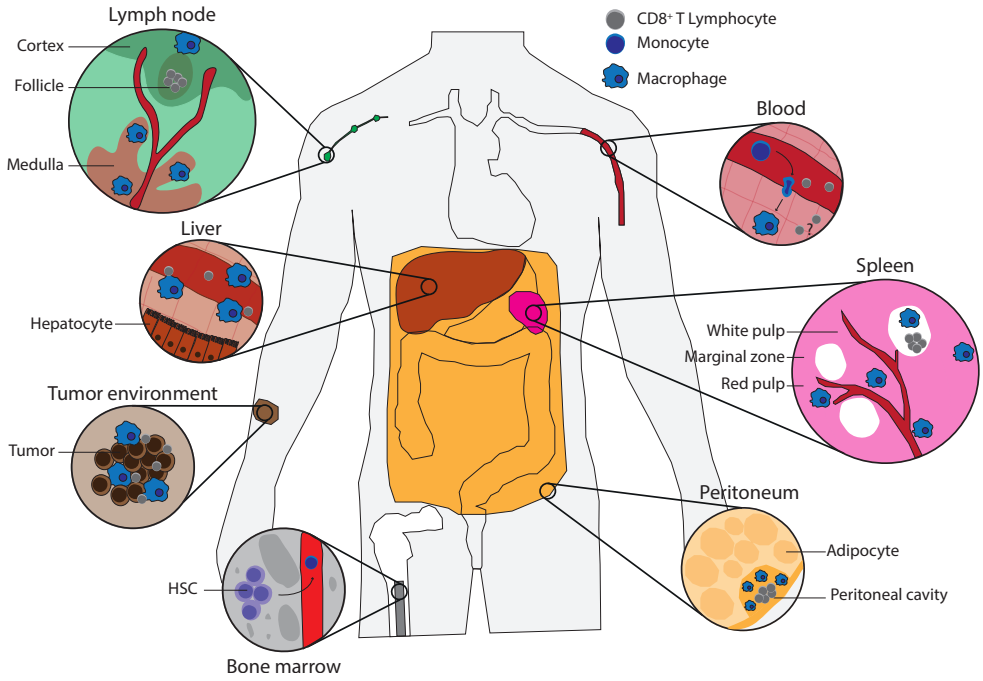

Fig. 1 Macrophage types potentially capable of antigen cross-presentation. Populations of macrophages in various tissues of the body that can cross-present antigen and their potential interactions with $CD8^+$ T lymphocytes. Lymph node: blood vessel (red), cortex and follicle (green) with $CD8^+$ T lymphocytes (gray) in the T cell zone and subcapsular macrophages on the edge (blue), medulla (brown) including medullary sinus macrophages and medullary cord macrophages (blue), subcapsular macrophages and. Liver: blood vessel (red) containing $CD8^+$ T lymphocytes (gray) and Kupffer cells (blue) on the edge, which are surrounded by hepatocytes. Tumor environment: tumor-infiltrating macrophages (blue) and $CD8^+$ T lymphocytes (gray) in-between tumor cells (brown). Bone marrow: hematopoietic stem cells (HSCs), which give rise to monocytes (dark blue) that leave the bone marrow by blood vessels (red). Blood: Monocytes in the blood vessels (red) can extravagate through the vessel wall into tissues, where monocytes (dark blue) differentiate into macrophages (blue) and there could be memory T cells (gray) to re-activate upon infection. Spleen: the spleen consist of red pulp (pink), white pulp (soft pink) with follicles (white) containing $CD8^+$ T lymphocytes (gray) and blood vessels (red). The red and white pulp are separated by the marginal zone. All contain macrophages (blue), including the marginal zone, which contains marginal metallophilic and marginal zone macrophages. Peritoneum: the adipocytes (soft yellow) surround the peritoneal cavity (yellow) which includes large and small peritoneal macrophages macrophages (blue) and $CD8^+$ T lymphocytes (gray).

which are important in case of recurrent infections. Memory $CD8^+$ T lymphocytes are present in tissues such as in the skin (16), where they might encounter macrophages. Additionally, inflammatory macrophages may cross-present to re-activate effector $CD8^+$ T lymphocytes upon sustained infections in combination with production of IL-12 and IL-23, but independent of CD80, CD86, and CD28 co-stimulation (2). In contrast, cross-presentation by anti-inflammatory macrophages might have functions in immune tolerance against 'self' proteins, commensal microbes and food components, similar to the cross-presentation of immature DCs (17). The different roles of cross-presentation by macrophages possibly relate to the antigen source, type of macrophage and location of the macrophage as will be discussed below.

Cross-presentation by various macrophage types

Cross-presentation by splenic macrophages

In the spleen, different subsets of macrophages can be distinguished based on their anatomical location and function (Fig. 1). In mice the red pulp macrophages ($F4/80^{high}CD68^+VCAM1^+CD11b^{low}$) are involved in iron metabolism and clearance of senescent erythrocytes. The white pulp macrophages ($F4/80^{low}CD68^+$) are specialized in phagocytosis, for instance for clearance of apoptotic B cells, and for regulating immune responses against pathogens. The white and red pulps are separated by the marginal zone, and macrophages located in this area can recognize and clear blood-borne antigens. Marginal zone macrophages can be subdivided in the phenotypically and functionally different marginal metallophilic macrophages ($F4/80^{low}CD68^+CD169^+$), involved in virus clearance, and marginal zone macrophages ($F4/80^{low}CD68^+SIGN\text{-}R1^+$), involved in tolerance against apoptotic cells in blood (18). Given that both types of marginal zone macrophage encounter blood-borne antigens and the role of $CD169^+$ macrophages in $CD8^+$ T lymphocyte activation in the lymph nodes (see below), a role for these macrophages in $CD8^+$ T lymphocyte activation in the spleen is likely (19,20).

Evidence that splenic macrophages are capable of cross-presentation comes from the *in vitro* finding that isolated mouse splenocytes incubated with microspheres encapsulated with the model antigen ovalbumin could induce activation of ovalbumin-specific B3Z hybridoma cells (LacZ assay) (21). Macrophages might play a role in this $CD8^+$ T lymphocyte activation and it was not exclusively due to splenic DCs, since ovalbumin-specific T cell activation (*in vivo* cytotoxicity assays) was still observed with splenocytes from mice that were depleted of $CD11c^+$DCs (21). Moreover, splenocytes from mice depleted of both $CD11c^+$DCs and $CD11b^+$macrophages showed less T cell activation compared to mice depleted of $CD11c^+$DCs only, suggesting that macrophages can cross-present in the absence of DCs (21). However, the ovalbumin-containing microspheres used in this study were specifically designed for vaccination and thereby might enhance cross-presentation also in cell types that normally are

not (or less) able of this process. Moreover, the physiological relevance of this finding is unclear, because CD11c and CD11b expression alone seems not sufficient to distinguish splenic macrophages and DCs (5). For instance, although depletion with CD11c will remove both $CD11c^{hi}CD11b^{+}CD8\alpha^{-}MHCII^{+}$ and $CD11c^{hi}CD11b^{-}CD8\alpha^{+}MHCII^{+}$ cDC subsets (22), the selection of CD11b is not sufficient to distinguish the various spleen macrophage populations since this would require selection on CD169 or SIGN-$R1^{+}$ (5,22). Moreover, this isolation method might result in contamination of the $CD11c^{+}$ DC population with $CD11c^{int}F4/80^{high}$ red pulp macrophages (23).

Recently, these $CD11c^{int}F4/80^{high}$ red pulp macrophages became of interest since, similar to DCs, they express CD11c and their location in the spleen allows them to acquire blood-borne antigens (23). Indeed, *in vitro* cross-presentation of fluorescently-labelled ovalbumin by murine $CD11c^{int}F4/80^{high}CD4^{-}CD8^{-}CD11b^{-}CD80^{+}CD86^{+}MHCII^{+}Gr1^{-}MARCO^{-}$ red pulp macrophages resulted in faster OT-I cell proliferation and higher expression of the T cell activation markers granzyme-B, TNF-α and production of IFN-γ than with the $CD11c^{high}CD8\alpha^{+}DEC205^{+}$ cDC1 subset (23). These OT-1 cells activated by the red pulp macrophages did not express activation markers CD127, KLRG1 and CX3CR1, suggesting they were so-called early effector cells which do not develop into memory cytolytic T cells (23,24). In contrast to the cDC1 cells, uptake of the model antigen ovalbumin by the red pulp macrophages relied on the mannose receptor CD206 (23). To address the role of cross-presentation by red pulp macrophages *in vivo*, mouse negative for the transcription factor SpiC were studied (23). SpiC mice lack $CD11c^{int}F4/80^{high}$ red pulp macrophages, as SpiC is required for their development, while cDC1 DCs are unaltered because they rely on the transcription factor Batf3 (23,25). Infection of SpiC knockout OT-1 mice with AdLGO adenovirus expressing ovalbumin resulted in a higher initial viral load, while viral clearance by ovalbumin-recognizing OT-I cytolytic T cells was achieved after ten days. In contrast, cDC1 deficient Batf3 knockout mice showed no changes in the initial viral load, but viral clearance was delayed until day 14 (23). In line with the $KLRG1^{-}CX3CR1^{-}$ phenotype of the cytolytic T cells induced by red pulp macrophages *in vitro*, *in vivo* reinfection experiments in SpiC knockout mice showed that red phase macrophages are not essential for induction of memory cytolytic T cell responses (23). These findings suggest that cross-priming by red pulp macrophages is necessary to contain early viral spread by triggering a fast but short antiviral response, whereas the main function of cDC1 cells is cross-priming of cytolytic T cells for complete viral clearance and development of memory cytolytic T cells.

The cross-presentation capabilities of other splenic macrophages have also been investigated *in vivo*. First, in mice with melanoma that underwent thermal ablation, isolated $CD11b^{+}CD11c^{-}CD45^{+}$ splenic macrophages were shown capable of cross-

presentation as directly shown with a fluorescently-labelled antibody recognizing MHC-I (H-2Kb-) loaded with the ovalbumin-derived epitope SIINFEKL (26). In addition, the $CD8^+$ lymphocytes in the tumor were specific for the antigen as shown by MHC-I tetramers carrying this epitope (26). Second, increased numbers of $CD169^+F4/80^+CD11b^+CD45^+$ macrophages were observed in the treated tumor region and spleen, which might suggest a role for these $CD169^+$ macrophages in $CD8^+$ T lymphocyte activation for elimination of the tumor (26). In line with a role for macrophages in cross-presentation, depletion of all phagocytosing cells using clodronate liposomes showed that after seven days, when the DCs already repopulate while the spleen macrophages need two weeks, the $CD11c^+$ DCs were unable to activate OT-I cells (IFN-γ, ovalbumin-specific cytotoxicity) with ovalbumin targeted by an antibody to the Siglec-1 receptor (CD169) *in vitro* (27). In contrast, direct targeting to $CD8^+$ DCs via ovalbumin conjugated to an antibody recognizing CD205 resulted in efficient activation of $CD8^+$ T lymphocytes *in vitro* (27), indicating that directly targeted antigen was cross-presented by the DCs.

However, a role for DCs in activation of cytolytic T cells by macrophages cannot be excluded, as the antigen might be transferred from the macrophages to the DCs (26). Thus, although for instance ovalbumin targeted to the receptor Siglec-1 is ingested by marginal metallophilic macrophages, it might be subsequently transferred to $CD8^+$ DCs for cross-presentation to $CD8^+$ T lymphocytes (27). $CD8^+$ DCs reside not only in the white pulp, but also locate the red pulp and marginal zone (28), and in principle they could encounter marginal metallophilic macrophages residing in the latter area. Migration of the DCs to the marginal zone might be important for antigen transfer, because treatment of mice with pertussis toxin, which blocks chemokine receptor mediated migration, no longer resulted in OT-I $CD8^+$ T lymphocyte proliferation using ovalbumin targeted to Siglec-1 (27). In contrast, $CD8^+$ T lymphocyte responses after direct targeting of ovalbumin to $CD8^+$ DCs via CD205 were unaffected by treatment with pertussis toxin (27). The $CD169^+$ macrophages and $CD8^+$ DCs have also been visualized in close interaction with each other using immunofluorescence microscopy and flow cytometry. In this case, the $CD169^+$ macrophages were suggested to move to the $CD8^+$DCs, although this was not specifically shown (29). Moreover, the specific antigen transfer seems to depend on the sialoadhesin CD169, which is involved in cell adhesion, since the use of Sn-KI mice, which expresses a mutated form of CD169, resulted in a reduced amount of antigen positive $CD8^+$ DCs (fluorescent ovalbumin) and a reduced amount of ovalbumin-specific IFN-γ producing $CD8^+$ lymphocytes (29). The immune stimulus of $CD169^+$ macrophages is also important, since *in vivo* administration of liposomes targeted to CD169L, present on $CD169^+$ macrophages, and containing ovalbumin and a TLR7 agonist induced expression of the activation markers CD86 and CD80 in the $CD169^+$ macrophages and induced *in vivo* OT-I $CD8^+$

T lymphocyte activation measured by proliferation, whereas only OT-II $CD4^+$ T cell responses were observed in absence of the TLR7 agonist (30). Altogether, it seems that murine $CD169^+$ macrophages in the spleen contribute to cross-presentation either directly or by transferring antigens to $CD8^+$ DCs in the spleen.

Cross-presentation by lymph node macrophages

According to their anatomical location in the lymph nodes and expression of surface markers, lymph node macrophages can be divided into subcapsular sinus macrophages (SSM; $F4/80^-CD169^+$), medullary sinus macrophages (MSM; $F4/80^+CD169^+$) and medullary cord macrophages (MCM; $F4/80^+CD169^-$) (31) (Fig. 1). Many lymph node macrophages are directly exposed to lymph fluid (31), which in principle enables them to efficiently take up lymph-borne antigens to present to T cells (31).

In vivo evidence that $F4/80^+CD169^+$ MSMs and $F4/80^+CD169^-$ MCMs are capable of cross-presentation, comes from the finding that only these cells can induce anti-tumor effects through stimulation of tumor-specific $CD8^+$ T lymphocytes when targeted by a nanogel packed with tumor-specific synthetic long peptide antigen (LPA) and a TLR9 agonist (32). In this study, isolated $F4/80^+CD169^+$ MSMs, 18 hours after injection of the nanogel, were most efficient at inducing LPA-recognizing DUC18 $CD8^+$ T cell responses *in vitro* measured by IFN-γ production and proliferation, which is possibly caused by a higher expression of CD80 and CD86 when compared to MCMs (32). Moreover, DCs and $F4/80^-CD169^+$ SSMs did not induce T cell responses and thereby it was concluded that were not essential for cytolytic T cell activation, probably because they did not have access to the nanogel. Injection of clodronate containing liposomes, which deplete macrophages but less DCs because they regenerate faster, blocked these antigen-specific $CD8^+$ T lymphocyte response in the lymph node (32). However, the physiological relevance of this finding is unclear, given that a highly artificial antigen was used, and it was not confirmed whether *in vivo* the macrophages actually processed the peptide for cross-presentation, presented it directly on MHC-I, or transferred it to DCs as described for splenic $CD169^+$ macrophages (27,29).

Other *in vivo* evidence that cross-presentation by $CD169^+$ macrophages might be important *in vivo*, is the finding that mice specifically depleted of $CD169^+$ SSM and MSM macrophages by induced expression of diphtheria toxin receptor (DTR) showed no OT-I $CD8^+$ T lymphocyte responses upon injection with ovalbumin expressing apoptotic cells or tumors (33). Additionally, $CD169^+CD11c^+$ macrophages were better cross-presenters compared to $CD169^+CD11c^-$ macrophages and $CD169^-CD11c^+CD8^+$ DCs, as shown by footpath stimulation with ovalbumin-expressing dead cells, followed by flow sorting of the DC and macrophage subsets and *in vitro* culturing with OT-I cells (proliferation, IFN-γ). Moreover, injection of $CD8α^+$ DCs in CD169-depleted mice did

not increase OT-I $CD8^+$ T lymphocyte responses (proliferation, IFN-γ) (33). Although this result at first glance seems strange given the essential role of $CD8\alpha^+$ cDCs in $CD8^+$ T lymphocyte activation (2), DCs are mostly located in the T cell zone upon arrival in the lymph node, where the antigens might possibly not be well accessible to the DCs, whereas the sinus location of the macrophages is beneficial for the antigen sampling (12). Additionally, microscopy revealed that $CD169^+$ macrophages and ovalbumin-specific OT-I $CD8^+$ T lymphocytes are in close contact already at four hours after antigen challenging, suggesting that the $CD8^+$ T lymphocytes re-localize to the sinus to be activated by antigen cross-presentation and then return to the T cell zone for proliferation (33,34). Thus, $CD169^+$ sinus macrophages might well activate $CD8^+$ T lymphocytes in the sinus, which would suggest that they are able of cross-presentation. This might explain the physiological relevance of these macrophages and their location in the sinus and not in the T cell zone (Fig. 1). Another option is that the $CD169^+$ SSM and MSM might transfer the antigen to DCs by CD169 to contribute to $CD8^+$ T lymphocyte activation, similar to $CD169^+$ macrophages in the spleen (see above) (27,29).

A role for $CD169^+$ macrophages in cross-priming is in line with *in vivo* mouse experiments with subcutaneous injection of antibodies targeting different receptor complexes where the model antigen ovalbumin is fused to IgG-binding domains of protein G (35). In this study, it was found that targeting to CD11c, CD40 and TLR2 (all present in dendritic cells) resulted in the most efficient *in vivo* cross-priming of OT-I T cells, while targeting to CD207 (expressed strongly by Langerhans cells) or CD169 (expressed by subcapsular sinus macrophages) still resulted in stronger cytolytic T cell responses than ovalbumin alone (i.e., without antibody targeting). In addition, microscopy showed that the protein complexes were ingested not only by $CD35^+$ follicular dendritic cells, but also by $CD169^+$ cells located in the marginal zone (35).

The cross-presentation capabilities have also been studied for macrophages from the tonsils. Although $CD11c^+HLA\text{-}DR^+CD14^+$ macrophages isolated from human tonsils efficiently ingested fluorescently-labelled necrotic cells, *in vitro* activation (IFN-γ) of the MelanA-recognizing LT12 $CD8^+$ T cell clone pulsed with a long peptide fragment of MelanA was much less efficient for macrophages than for $HLA\text{-}DR^+CD14^-BDCA1^+$ cDC2, $CD11c^+HLA\text{-}DR^+CD14^-BDCA3^+$ cDC1 and $CD11c^-HLA^-DR^+$ pDC tonsil DCs (36). Also, the *in vitro* cross-presentation of NS3 protein from hepatitis C to NS3 antigen specific $CD8^+$ T cell clones (IFN-γ) was less efficient for $CD11c^+HLA\text{-}DR^+CD14^+$ tonsil macrophages than for these DC subsets (36). These findings show that although tonsil macrophages might in principle be able to cross-present antigens, they are less capable than the major DC subsets.

Cross-presentation by liver macrophages

The macrophages in the liver are called Kupffer cells. This very heterogenous cell population (37) plays a major role in the clearance of gut-derived antigens and pathogens from the blood, making them in principle ideally positioned for cross-presentation to blood $CD8^+$ T lymphocytes. However, studying the Kupffer cells is challenging due to loss of cells during isolation and lack of consistent cell-membrane markers for sorting (37).

Murine hepatic cells cultured *in vitro* with the model antigen ovalbumin could induce proliferation of ovalbumin-recognizing Th1 HTL clone cells but not Th2 HTL cells (38,39). Although this cell mixture included Kupffer cells, the contribution of other hepatic cells, such as liver resident DCs, liver endothelial cells and hepatocytes, cannot be excluded. Indeed, a recent side-by-side comparison of ovalbumin-pulsed hepatocytes, Tie-2^+CD11b^{low} liver endothelial cells, and $CD11b^+F4/80^+$ Kupffer cells showed that all these cell types can induce OT-I $CD8^+$ T lymphocyte proliferation *in vitro* with similar efficiency as splenic $CD11c^+$DCs (40). However the physiological role in the initiation of cytolytic T cell responses by liver cells is unclear, because the levels of cytolytic T cell activation markers (CD44, CD25) and IFN-γ production were lower in the proliferated OT-I $CD8^+$ T lymphocytes upon stimulation by the liver cells than upon stimulation with $CD11c^+$ DCs (40).

Interestingly, Kupffer cells seem to suppress immune responses for immune tolerance as seen in murine transplantation studies by rejection of allogeneic liver transplants (41). Injection of CFSE labelled OT-I $CD8^+$ lymphocytes in the portal vein and intraperitoneal injection of ovalbumin peptide, resulted in retention of activated OT-I $CD8^+$ cells (proliferation and CD45 expression) in the liver, followed by their deletion, probably by apoptosis as shown by DNA fragmentation (42). This apoptosis seems to be dependent on interactions with the Kupffer cells since CSF-1 deficient mice, which have no mature developed tissue macrophages since CFS-1 is essential for their differentiation, resulted in an increased presence of activated OT-I $CD8^+$ lymphocytes(proliferation and CD45 expression) (42). Thus, it seems that Kupffer cells are able of cross-presentation and although the physiological relevance of this is still unclear, this might play a role in immune tolerance.

Cross-presentation by tumor-infiltrating macrophages

The immune system can either combat cancer by immunosurveillance where the cancer is recognized and cross-presented by antigen presenting cells (43) or promote tumor progression via (in)direct suppression of $CD8^+$ T lymphocytes and other immune cells (43,44). When directly comparing cross-presenting capacities of DCs and primary macrophages isolated from human peritoneal tumor ascites *in vitro*, it was found

that HLA-DR$^+$CD11c$^+$CD1c$^-$CD16$^+$ tumor macrophages even more effective than HLA-DR$^+$CD11c$^+$CD1c$^+$CD16$^-$ DCs at cross-presentation to the LT12 CD8$^+$ T cell lines (IFN-γ) recognizing the tumor-specific antigen MelanA, which does not require co-stimulation for activation (45). Moreover, thermal ablation of ovalbumin-expressing melanoma in mice resulted in increased cross-presentation of ovalbumin-derived epitopes by intratumoral CD11b$^+$CD11c$^-$ macrophages as directly measured by an antibody that recognizes MHC-I loaded with SINFEKL (26). Additionally, this cross-presentation was concomitant with an increased presence of ovalbumin-specific CD8$^+$ T lymphocytes (26). Although these findings support a role for macrophages in ovalbumin-specific CD8$^+$ T cell responses, evidence shows that DCs are essential in the initial activation of the naive CD8$^+$ T lymphocytes.

Although human ascites monocyte-derived CD1a$^+$CD16$^+$ macrophages efficiently presented an MelanA epitope to the LT12 CD8$^+$ T cell clone *in vitro,* cross-priming of allogeneic naive cytotoxic CD8+ T cells (proliferation and expression of granzyme A, perforin, IFN-γ) was only observed by ascites monocyte-derived DCs and not ascites-monocyte-derived macrophages (45). The reason for this is that the ascites monocyte-derived macrophages lacked expression of co-stimulatory signals such as CD80 and CD86 and produced almost no IL-12, whereas these were expressed by the ascites monocyte-derived DCs (45). These findings indicate that although tumor-infiltrating macrophages are capable of cross-presentation, they do not provide the co-stimulatory signals required for cross-priming of cytolytic T cells.

More indirect evidence on cross-presentation by tumor infiltrating macrophages was obtained in a microscopy study in mice showing that migrating OT-I CD8$^+$ T lymphocytes in ovalbumin-expressing tumors can have long interactions with F4/80$^+$ macrophages, which might suggest activation by cross-presentation (46). Furthermore, CD8$^+$ T lymphocytes might be activated for virus peptide by macrophages, because tumors in mice caused by injected fibrosarcoma cells transfected with lymphocytic choriomeningitis virus (MCA102(gp33)) were infiltrated by CD8$^+$ T lymphocytes, a few CD11c$^+$ DCs and a high number of CD11b$^+$ macrophages (44). However, these tumor-infiltrating CD8$^+$ T lymphocytes exhibited a highly activated phenotype (up-regulated CD25, CD44 and CD69 and down-regulated CD62L expression) but lacked effector cell function, measured by a killing assay, suggesting modulation of the CD8$^+$ T lymphocytes by the tumor environment. The tumor-infiltrating CD11b$^+$ macrophages were also able to efficiently cross-present to tumor infiltrating CD8$^+$ T lymphocytes (proliferation, IFN-γ, cytolytic activity) for gp33 *in vitro* (44). However, the co-stimulatory molecules CD80, CD86 and ICAM-1 were not expressed in these macrophages, which could explain the observed loss of the killing by the CD8$^+$ T lymphocytes (44).

Cellular pathways of cross-presentation by macrophages

In DCs, antigen cross-presentation can be the result of two distinct cellular pathways: the cytosolic and vacuolar pathway (2,47). In the cytosolic pathway, proteins are first transported from the lumen of the endosomal compartment to the cytosol for degradation by the proteasome. Subsequently, the derived peptides can be processed via the MHC-I presentation pathway. The peptides are relocated via the transporter associated with antigen presentation (TAP) into the ER, where they are processed by ER aminopeptidases (ERAP), or brought back into the antigen containing endosomes to be processed by insulin-regulated aminopeptidase (IRAP). The loading of peptides on MHC-I occurs within these compartments (2,17). The other main cross-presentation pathway is the vacuolar pathway, where proteins are processed by endo/lysosomal proteases, such as cathepsin S, and loaded on MHC-I within the endo/lysosomal compartments (2,17). As we will discuss below, evidence suggests that the vacuolar pathway seems to be predominantly used by macrophages, although they might be able to also cross-present via the cytosolic pathway.

Bone marrow and blood monocyte-derived macrophages cross-present via the vacuolar pathway

Monocytes derived from haematological precursors in the bone marrow (BM) can migrate via the blood stream to other tissues where they can differentiate into macrophages to perform tissue specific functions, eliminate pathogens or restore tissue homeostasis (48). Therefore, for research on mouse macrophages, stem cells are frequently isolated from the BM and differentiated into macrophages *in vitro*. For research on human macrophages, $CD14^+$monocytes are often isolated from the blood. Both are then differentiated into macrophages *in vitro,* either by the use of macrophage colony stimulating factor (M-CSF), which results in a homogenous macrophage population, or by the use of granulocyte-macrophage (GM)-CSF, which results in a cell population reflecting resident macrophages (6). However, GM-CSF is also used to differentiate DCs from monocytes, therefore the macrophage culture might contain DCs (5,6). Thereby, it should be kept in mind that *in vitro* cultured monocyte-derived macrophages and monocyte-derived DCs have similarities (5,6) and that both *in vitro* BM-derived and monocyte-derived macrophages and DCs are artificial cell populations that might not be identical to their *in vivo* murine and human counterparts (Fig. 1).

The cross-presenting capacities of BM-macrophages seem to be higher than BM-DCs when tested side-by-side using liposomes encapsulating a mixture of the model antigen ovalbumin and the pore-forming protein sticholysin II. M-CSF differentiated BM-macrophages were better in activating the ovalbumin-recognizing B3Z $CD8^+$ T cell line (which does not require co-stimulation) than GM-CSF differentiated BM-

derived DCs *in vitro* (49). This could be explained by the lower ability of the DCs to internalize the antigen-containing liposomes (49). Furthermore, inhibitors of the lysosomal proteases cathepsins and leupeptin resulted in a reduced efficiency of B3Z T cell activation by the BM-macrophages, whereas a proteasome inhibitor (epoxomicin) had no effect (49). This suggests that ovalbumin is processed for cross-presentation in lysosomes instead of the cytosol and therefore that cross-presentation occurs via the vacuolar pathway in BM-derived macrophages.

In line with the conclusion that BM-derived macrophages cross-present via the vacuolar pathway, the proteasome inhibitor lactacystin did not inhibit cross-presentation of FITC-labelled ovalbumin peptide complexed with heat shock protein to CD8OVA1.3 T hybridoma cells (colorimetric bioassay for IL-2) in M-CSF differentiated BM-macrophages, activated 48h with IFN-γ, whereas there was only a slight reduction in activation when TAP-deficient macrophages were used (50). In contrast to BM-macrophages, proteasome inhibition resulted in a marked reduction of CD8OVA1.3 T hybridoma cell activation by GM-CSF differentiated BM-DCs (50), suggesting that these macrophages and DCs use different pathways for cross-presentation. Final evidence that BM-macrophages employ the vacuolar pathway for cross-presentation comes from the finding that cross-presentation of ovalbumin to B3Z T cells by MAC-1$^+$ BM-macrophages was reduced by a peptide aldehyde inhibitor that also inhibits lysosomal proteases (LLnL), whereas a proteasome inhibitor (LLM) had no effect and resulted in normal B3Z T cell activation (51). Thus, whereas BM-DCs can use the cytosolic route of cross-presentation, antigens seem to be predominantly cross-presented by BM-macrophages via the vacuolar pathway. This would explain why BM macrophages have lower expression and activity of the NADPH oxidase NOX2, which is essential for cross-presentation via the cytosolic pathway (52,53).

Less data is available on cross-presentation mechanisms in human monocyte-derived macrophages. They can also cross-present via the vacuolar pathway similar to murine BM-macrophages, because an *in vitro* study showed that the proteasome inhibitor lactacystin did not impair cross-presentation by human blood monocyte-derived CD1a$^+$CD16$^+$ macrophages of MelanA antigen to CD8$^+$T cell LT12 clones (IFN-γ), whereas lysosomal cysteine protease inhibition (with a pan-cathepsin inhibitor) impaired activation of this T cell line (45). These results support that monocyte-derived macrophages can cross-present via the vacuolar pathway, which might explain why lysosomal proteases are expressed in higher levels in human monocyte-derived CD1a$^+$CD16$^+$ macrophages compared to monocyte-derived CD1a$^+$CD14$^+$ DCs (45). However, for various HIV-1 epitopes, evidence suggests that monocyte-derived macrophages can cross-present via both the cytosolic and vacuolar pathways. Proteasome inhibition by MG132 or epoxomicin did not completely block activation

(IFN-γ) of epitope-specific $CD8^+$ T lymphocyte clones by LPS and R848 matured monocyte-derived macrophages, suggesting both proteasome-dependent and -independent processing, although inhibition of lysosomal cysteine proteases by E64 did not affect cross-presentation (54). In line with this notion that human monocyte-derived macrophages might cross-present via both the cytosolic and vacuolar pathways, similar to DCs, is that the difference in expression levels of NOX2, required for the cytosolic route of cross-presentation, is less clear for human monocyte-derived macrophages and DCs (55) than for murine BM-macrophages and BM-DCs (52).

Besides the macrophage types described so far, a new murine $F4/80^+$ subset of antigen presenting cells was described containing characteristics of both macrophages ($CD64^+MERTK^+$) and cDC2s ($CD11c^{hi}MHCII^{hi}CD11b^+CD24^+CD64^+CD169^+$), while being from mouse monocyte origin (56). These hybrid DC-macrophages have been found in multiple tissues including lymph node and spleen, and they seem increased in the tumor microenvironment. Supporting a functional role in the tumor environment, these cells are efficient at B16 tumor cell uptake (GFP-labelled)_and *in vivo* blocking of CSF1R-positive cells (blocking most macrophages, including the new DC-macrophage hybrid subset) significantly decreased antigen cross-presentation to ovalbumin-specific OT-I $CD8^+$ T lymphocytes (proliferation) (56). Moreover, when isolating these DC-macrophage hybrid cells from lymph nodes of mice inoculated with ovalbumin-expressing cancer cells and *in vitro* culturing them with OT-I $CD8^+$ T lymphocytes, efficient T cell proliferation was observed (56).

Splenic red pulp macrophages cross-present via the cytosolic pathway

As discussed above, both *in vitro* and *in vivo* evidence indicate that murine $CD11c^{int}F4/80^{high}$ red pulp macrophages from spleen are capable of cross-presentation (23). At least for the model antigen ovalbumin, evidence shows that they process this antigen via the cytosolic pathway. Following its uptake via the mannose receptor CD206, microscopy showed that fluorescently-labelled ovalbumin colocalizes with the mannose receptor to early endosomes but not to late endosomes in $CD11c^{int}F4/80^{high}$ red pulp macrophages (23). *In vitro* cross-presentation to OT-I T cells was almost completely blocked by the TAP-inhibitor UL49 and the proteasome inhibitor epoxomicin (23), indicating that red pulp macrophages cross-present via the cytosolic pathway(23).

Peritoneal macrophages cross-present via both cellular pathways

There are two subsets of peritoneal macrophages: large peritoneal macrophages ($F4/80^{high}$, $MHC\text{-}II^{low}$) which play a role in maintaining homeostatic conditions in the peritoneal cavity and represent an anti-inflammatory type, and small peritoneal

macrophages (F4/80^{low}, MHCII^{high}) which are important during inflammation (57) (Fig. 1). The large peritoneal macrophages express higher levels of TLR-4 and co-stimulatory molecules (CD80/CD86/CD40). Likely, both peritoneal macrophage types can cross-present (58–61), but there is contrasting evidence whether peritoneal macrophages use the vacuolar or cytosolic pathway. The yield of macrophages from the peritoneum is low, therefore mice are often pre-stimulated with thioglycolate, which recruits immature macrophages into the peritoneum. Thioglycolate isolated peritoneal macrophages resemble mostly small peritoneal macrophages, but have an atypical morphology and function that is not consistent with the phenotype of tissue macrophages, but more with monocyte-derived macrophages (62). Moreover, during the isolation they can come in contact with LPS from the used broth, which is ingested by the macrophages, and as a result they are able to respond to INF-γ priming without any other stimulation (63). A better option for isolation of peritoneal macrophages for cross-presentation and phagocytosis studies is called Bio-Gel-elicted macrophages isolation, which makes use of Bio-Gel beads that cannot be phagocytosed (63).

The angiotensin-converting enzyme (ACE) 10/10 mice overexpress the peptidase ACE, which is a peptidase normally located within the endoplasmic reticulum (ER) and involved in generation of MHC-I epitopes. ACE is also involved in cross-presentation probably following its transfer antigen-containing phagosomes or endosomes (2,61). The injection of ovalbumin-pulsed peritoneal macrophages isolated from ACE 10/10 mice (using thioglycolate) and ovalbumin-specific OT-1 CD8$^+$ T lymphocytes into a wildtype mouse resulted in proliferation of the CD8$^+$ T lymphocytes (61). Moreover, upregulation of the CD8$^+$ T lymphocyte activation marker CD69 occurred faster with peritoneal macrophages isolated from the ACE 10/10 mice, than with macrophages isolated from wildtype mice (61). Although these studies rely on overexpression of ACE, these findings indicate that peritoneal macrophages in principle can cross-present antigen (61). Moreover, as ACE is potentially transferred from the ER to the lumen of antigen-containing endosomes (61), these findings suggest that peritoneal macrophages can cross-present via the vacuolar pathway.

More direct evidence that peritoneal macrophages can cross-present comes from the finding that a murine peritoneal macrophages cell line that recombinantly expresses interferon regulatory factor 7 (IRF7) was able to induce activation of ovalbumin-specific OT-I CD8$^+$ T lymphocytes *in vitro* (IL-2 production and CD40, CD80 and CD86 expression) (58). This activation could be inhibited by a proteasome inhibitor (lactacystin) (58), suggesting that the cytosolic pathway was used for cross-presentation of the antigen. Also the uptake by peritoneal macrophages of *Escherichia coli* in which ovalbumin was expressed could trigger activation (IL-2 production) of

the ovalbumin-recognizing B3Z CD8+ T cell line (60). These ingested *E. coli* bacteria remained in phagosomes that fused with lysosomes (60), suggesting processing of the bacteria for cross-presentation via the vacuolar pathway in this case.

Murine peritoneal macrophages also cross-presented virus-like particles containing the immunodominant epitope (p33) of lymphocytic choriomeningitis virus *in vitro* as observed by increased antigen-specific transgenic CD8+ T cell proliferation, but with a reduced efficiency compared to peritoneal isolated DCs (59). The DCs used both the TAP-dependent and independent pathway for cross-presentation (59), but there was no difference in activation of CD8+ T lymphocytes between TAP1-deficient peritoneal macrophages (thioglycolate stimulated) and wild-type controls, suggesting the use of the TAP-independent vacuolar pathway (59). Finally, murine peritoneal macrophages (thioglycolate stimulated) showed efficient cross-presentation of ovalbumin encapsulated in polylactic-co-glycolic acid particles to the B3Z CD8+ T cell line *in vitro*, and these particles resided in LAMP1-positive lysosomes even 48 hours after phagocytosis (64). Based on this observation, it was suggested that the antigenic proteins might be translocated from the endo/lysosomal compartments to the cytosol. However, it might also be possible that the antigen is processed by endo/lysosomal proteases and cross-presentation occurs via the vacuolar pathway.

To target cross-presentation by peritoneal macrophages, the effect of downregulation of various signaling pathways has been investigated. Compared to macrophages isolated from wildtype mice, CD11b+F4/80+ peritoneal macrophages (thioglycolate stimulated) from signal transducer and activator of transcription (STAT) 3 knockout mice were able to stimulate more potent OT-1 CD8+ T lymphocyte proliferation and IFN-γ production *in vitro* with irradiated tumor cells expressing ovalbumin (65). Similar observations were made with a different antigen derived from the influenza protein hemagglutinin in combination with hemagglutinin-recognizing CD8+ T lymphocytes isolated from CLN4 transgenic mice (65). Since STAT3 is activated by inflammatory cytokines, such as IL-6, this finding indicates that cross-presentation by peritoneal macrophages is influenced by the tissue environment. The increase in ovalbumin-recognizing CD8+ T lymphocyte activation was not seen for STAT4 or 6 knockout mice (65). Additionally, the antigen VSV8 in a complex with heat shock protein gp96 was more efficiently cross-presented (cytotoxicity by ^{51}Cr release) to VSV-specific cytotoxic T cells *in vitro* by mice peritoneal macrophages compared to the antigen alone (66), again indicating that the form of the antigen plays an important role for cross-presentation by peritoneal macrophages.

So, although these results clearly show that peritoneal macrophages are able of cross-presentation *in vitro*, the *in vivo* relevance remains unclear because of the highly artificial nature of the antigen and the lacking definition of the used peritoneal macrophage subset.

Concluding remarks

As discussed in this review, many macrophage types seem capable of antigen cross-presentation with similar, or even better, efficiency as DCs. Moreover, macrophages seem to employ mostly the vacuolar pathway of cross-presentation while DCs use both the vacuolar and cytosolic pathway. Especially the cross-presenting abilities of macrophages in the spleen, liver and lymph nodes might be physiologically relevant, because they have easy access to blood-borne antigens, whereas for the DC this access might be restricted. However, the role of macrophages in $CD8^+$ T lymphocyte activation should be further investigated, since $CD169^+$ macrophages might not activate the $CD8^+$ T lymphocytes themselves but could transfer antigen to DCs by CD169 (20). The role of $CD169^+$ macrophages should therefore be further investigated, and it should be investigated if they directly cross-present to $CD8^+$ T lymphocytes *in vivo* or transfer the antigen to DCs (27,29).

As apparent from this review, the *in vivo* roles and cellular pathways of macrophage cross-presentation are mostly unknown. One important reason for this is that the isolation of the specific macrophage types is technically challenging because (i) surface markers overlap resulting in contamination with other cell types and (ii) low numbers of primary macrophages can be isolated (63). Moreover, the available *in vivo* research on cross-presentation by macrophages is almost exclusively focused on the effect of vaccination strategies, such as liposomes encapsulating antigen, where $CD8^+$ T lymphocyte activation markers and/or proliferation are used as the sole measures of cross-presentation. Although $CD8^+$ T lymphocyte activation and proliferation depend on cross-presentation, they also depend on other factors such as co-stimulation by cytokines (e.g. IL-12) and co-stimulatory receptors (e.g. CD80, CD86) (2). This co-stimulation is generally stronger present in DCs than macrophages, and it is particularly absent in the alternatively-activated anti-inflammatory macrophages. Using T cell activation as the sole readout might thereby result in overlooking cross-presentation capabilities in macrophage types that do not provide co-stimulation, whereas this might have important roles for instance in maintenance and/or restoration of immune tolerance. Cross-presentation by immature/inactivated DCs has been suggested to allow maintenance of tolerance (67), and it might well be that cross-presentation by Kupffer cells and/or anti-inflammatory macrophages plays a similar role (41,42). Therefore, the macrophage cross presenting capabilities and pathways should be further elucidated, for instance *in vitro* by exposing the cells to

antigens and measuring cross-presentation directly using antibodies (26,27) followed by measuring their ability to activate $CD8^+$ T cell lines that do not need co-stimulation. However, since environmental factors might be needed, these *in vitro* experiments should be performed in parallel with *in vivo* experiments. To determine if a particular macrophage type is needed for efficient $CD8^+$ T lymphocyte activation in *vivo*, this macrophage type could be depleted using DTR-mice as previously explained (68,69). However, this relies on surface marker expression and is not effective if the targeted macrophage type does not express an unique surface marker.

It is increasingly clear that not only DCs and macrophages can cross-present antigens, but many other endocytic cell types are capable of cross-presentation as well, including monocytes (70,71), B cells (72), neutrophils (73) and endothelial cells (74). The physiological roles of cross-presentation by these diverse cell types are still unclear, but it seems likely that this also allows the potentiation of $CD8^+$ T lymphocyte responses or the maintenance or restoration of immune tolerance.

Lastly, studies on cross-presentation have focused on direct $CD8^+$ T lymphocyte activation, but the potential role of tissue macrophages in development and reactivation of memory $CD8^+$ T lymphocytes has hardly been investigated. Although $CD11c^{int}F4/80^{high}$ splenic red pulp macrophages are not essential for development of memory $CD8^+$ T lymphocytes (23), it might well be that other macrophage types are involved in this process or that macrophages are capable of reactivation of memory $CD8^+$ T lymphocytes upon recurrent immune challenges (75,76). Recently, there is a growing interest in the activation of memory T cells in the tissues and lymphatic system, which can confer rapid host protection upon cognate antigen-mediated activation and results in direct killing of infected cells (75,76). Especially tissue-resident macrophages might be important in the development and reactivation of tissue-resident memory $CD8^+$ T lymphocytes by rapid and local cytokine secretion during re-infection (74). It is increasingly clear that macrophages can provide factors such as chemokines and cytokines that regulate the localization, differentiation and survival of tissue-resident memory T cells (73). Moreover, monocyte-derived antigen presenting cells provide TNF superfamily costimulatory signals, which substantially increase the formation of tissue-resident memory T cells during viral infection (73).

Since macrophages can cross-present and thereby might aid in $CD8^+$ T lymphocyte responses, stimulating macrophages to cross-present might be a promising strategy for anti-tumor or anti-viral therapies.

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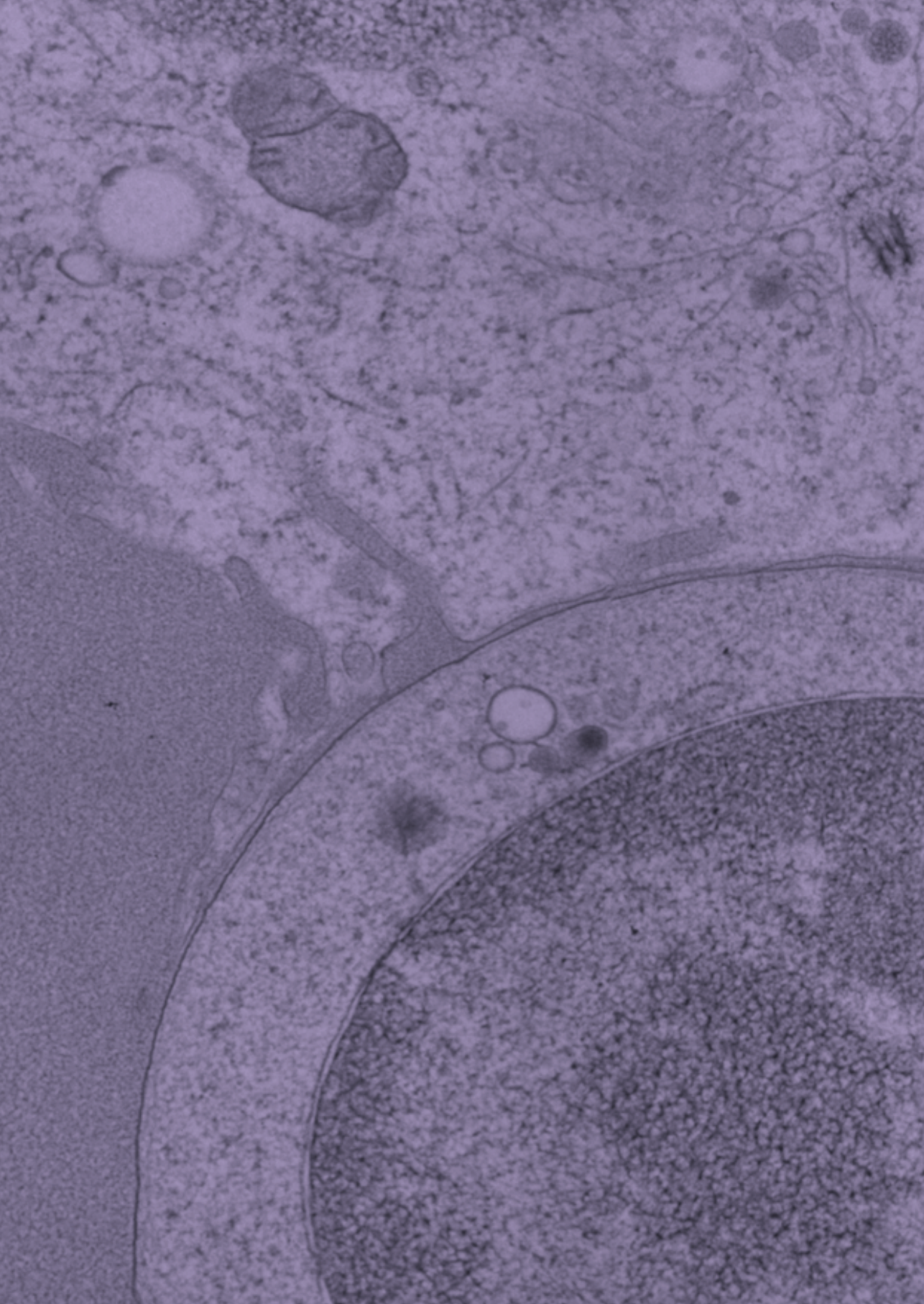

Chapter 6

Reverse signaling by MHC-I molecules in immune and non-immune cell types

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Abstract

Major histocompatibility complex (MHC) molecules are well-known for their role in antigen (cross-) presentation, thereby functioning as key players in the communication between immune cells, for example dendritic cells (DCs) and T cells, or immune cells and their targets, such as T cells and virus-infected or tumor cells. However, much less appreciated is the fact that MHC molecules can also act as signaling receptors. In this process, here referred to as reverse MHC class I (MHC-I) signaling, ligation of MHC molecules can lead to signal-transduction and cell regulatory effects in the antigen presenting cell. In the case of MHC-I, reverse signaling can have several outcomes, including apoptosis, migration, induced or reduced proliferation and cytotoxicity towards target cells. Here, we provide an overview of studies showing the signaling pathways and cell outcomes upon MHC-I stimulation in various immune and non-immune cells. Signaling molecules like RAC-alpha serine/threonine-protein kinase (Akt1), extracellular signal-regulated kinases 1/2 (ERK1/2) and nuclear factor-κB (NF-κB) were common singaling molecules activated upon MHC-I ligation in multiple cell types. For endothelial and smooth muscle cells, the *in vivo* relevance of reverse MHC-I signaling has been established, namely in the context of adverse effects after tissue transplantation. For other cell types, the role of reverse MHC-I signaling is less clear, since aspects like the *in vivo* relevance, natural MHC-I ligands and the extended downstream pathways are not fully known. The existing evidence, however, suggests that reverse MHC-I signaling is involved in the regulation of the defense against bacterial and viral infections and against malignancies. Thereby, making reverse MHC-I signaling a potential target for therapies against viral and bacterial infections, cancer immunotherapies and management of organ transplantation outcomes.

Introduction

Major histocompatibility complex class I (MHC-I) molecules are present on all nucleated cells and are classically known for presenting peptides derived from endogenous antigens to cytotoxic $CD8^+$ T lymphocytes (CTL). Additionally, in the case of professional antigen presenting cells (APCs) (i.e. dendritic cells (DCs), macrophages and B cells), MHC-I can also present exogenous antigens through a process called antigen cross-presentation. Moreover, MHC-I molecules are important immune regulators, since their expression levels regulate activation of natural killer (NK) cells (1,2).

An underappreciated function of MHC-I molecules is their ability to act as signaling receptors. In this process, here referred to as reverse MHC-I signaling, ligation of MHC molecules can lead to signal-transduction and cell regulatory effects in the APC (3,4). Multiple studies have shown that reverse MHC-I signaling can influence processes like cell activation, proliferation, maturation, cytotoxicity and migration, or even lead to cell anergy and apoptosis (3,5,6). MHC-I reverse signaling has been observed in multiple cell types, ranging from immune cells, such as macrophages, NK cells, T cells and B cells, to non-immune cells like endothelial and smooth muscle cells (7,8). Furthermore, reverse MHC-I signaling has been investigated in the context of viral and bacterial infections (6,9), transplantation immunity (10), malignancies (11) and brain development (12). Here, we review the evidence for the alternative role of MHC-I as reverse signaling molecules across immune and non-immune cells.

MHC-I function in T cell activation and NK cell regulation

MHC molecules (referred to as human leukocyte antigen (HLA) in humans and histocompatibility system 2 (H-2) in mice) play an important role in the communication between the innate and adaptive immune system. There are two classes of MHC molecules that are involved in antigen presentation: MHC class I and MHC class II. MHC-I molecules are present on all nucleated cells and classically function to activate CTLs with endogenous antigens, whereas MHC-II molecules are present on professional antigen-presenting cells, and are involved in the activation of $CD4^+$ T cells with exogenous antigens (1). Exogenous antigens can also be presented by MHC-I to $CD8^+$ T cells, in a process called antigen cross-presentation, which is important for the defense against tumors and intracellular pathogens (13).

The interface formed between an APC and an antigen-recognizing T cell is called an immunological synapse. T cell activation requires the delivery of three molecular signals by the APC: First, the complex formed by MHC and the antigenic peptide is recognized by the T cell receptor (TCR) along with the co-receptor CD4 on helper T

cells (for MHC-II) or CD8 on CTLs (for MHC-I). Second, a costimulatory signal provided by CD80/86 is recognized by CD28 on the T cell. Third, the APC releases stimulatory cytokines that are recognized by their receptors on the T cell. Additionally, the immunological synapse is stabilized by adhesion molecules such as lymphocyte function-associated antigen (LFA)-1 and intercellular adhesion molecule 1 (ICAM-1). The combination of costimulatory signals and cytokines secreted by the APC determines the functional outcome of the interaction, such as the activation or inhibition of the T cell (14) (Figure 1A). The immunological synapse can be seen as a radially symmetric structure consisting of several supramolecular activation clusters (SMACs) (15). The classical synapse contains a central SMAC (cSMAC), where the MHC-TCR interactions, co-receptors (CD3 and CD4 or CD8) and costimulatory molecules are located. The surrounding peripheral SMAC (pSMAC) includes adhesion molecules LFA-1 and ICAM-1 and the distal SMAC (dSMAC) contains the transmembrane tyrosine phosphatase CD45, which activates signaling molecules promoting the T cell response (14,16,17). There are exceptions to this structure, for instance when T cells interact with DCs, where a multifocal synapse is formed (16,18).

In addition to their role in $CD8^+$ T cell activation, MHC-I molecules provide signals that are crucial for the regulation of different immune cells. For instance, NK cells carry inhibitory receptors that bind to MHC-I on the surface of other cells, independent of the antigen presented, thereby suppressing NK cell activation. This is particularly important as viruses and malignancies often downregulate MHC-I expression to circumvent T cell detection, requiring the action of NK cytotoxic function (2). In mice, one type of inhibitory receptors is the Ly49 family, which have a C-type lectin-like structure, with two transmembrane domains. Their functional homologs in human are the killer Ig-like receptors (KIR), which belong to the Ig-superfamily. These two families have similar inhibitory functions despite their differences in structure and evolutionary origin. Additionally, in both species the C-type lectin-like molecules CD94 and NKG2 (CD94/NKG2) form heterodimers with inhibitory properties (2). Another important group of MHC-I receptors are the Leukocyte Immunoglobulin-Like Receptors (LILRs), also known as CD85. They can have both activating and inhibitory roles, and have been found in immune (e.g. lymphocytes, neutrophils, eosinophils, macrophage, DCs) and non-immune cells (e.g. endothelial cells) (19).

The alternative role of MHC-I as signaling molecules and their structure

Besides their role in antigen presentation for T cell activation, both MHC-I and MHC-II molecules are involved in reverse signaling, meaning that ligation of the MHC molecule leads to signal-transduction and cell regulatory effects in the APC (3). In this review we will focus on reverse MHC-I signaling, given the ample literature available

across different cell types, its importance for medical outcomes in transplantation patients, and its potential role regulating immune responses against infection and malignancies.

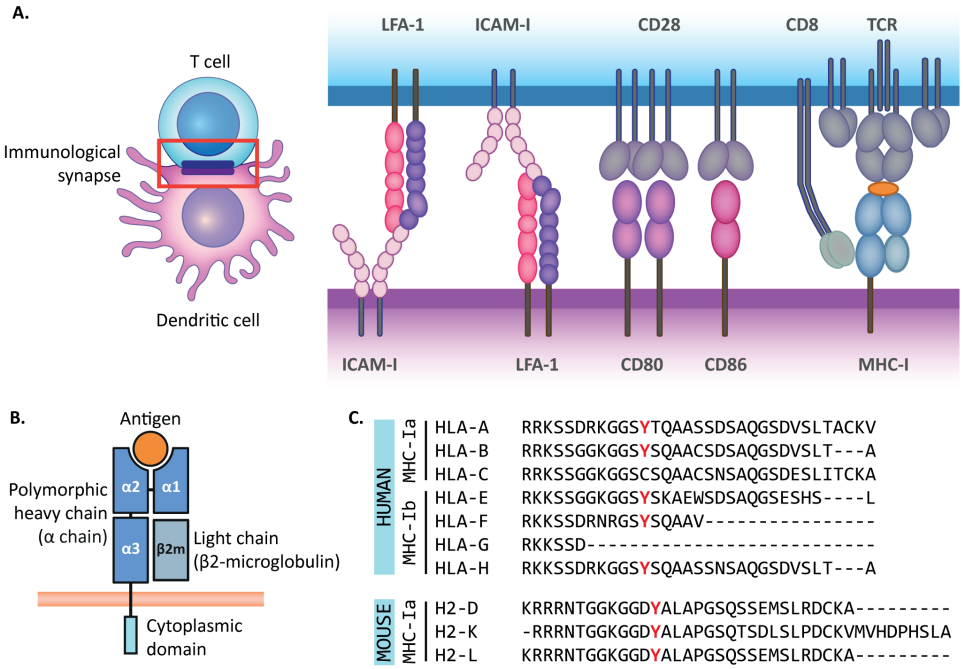

Figure 1. The antigen presenting role of MHC class I molecules and their structure. (A) The immunological synapse is a communication platform between a (professional) antigen-presenting cell and a T or NK cell. In the example presented here, MHC-I molecules on the surface of a dendritic cell (purple) present antigens (orange) for their recognition by the TCR of a $CD8^+$ T cell (blue). CD8 acts as co-receptor of the TCR for the recognition of the MHC-I molecule. At the immunological synapse (red box), other molecules required for the engagement and modulation of the interaction are also recruited, such as adhesion molecules (LFA-1 and ICAM-I) and costimulatory molecules (CD80/86 and CD28). **(B)** MHC-I molecules are heterodimers composed of a heavy α chain (blue) and a light chain (β2-microglobulin) (grey). Most α chains are made up of two peptide binding domains (α1 and α2), and an Ig-like α3 domain recognized by CD8 molecules on T cells. Additionally, the α chain has a transmembrane and a short cytoplasmic tail (cyan). **(C)** The cytoplasmic regions of several classical and non-classical MHC-I α chains contain a tyrosine residue that can be phosphorylated. This phospho-tyrosine residue has been proposed to interact with signaling proteins and thus lead to reverse signaling. Here we present the cytoplasmic regions of human classical (MHC-Ia) and non-classical (MHC-Ib) α chains, and the classical mouse α chains. However, some of the multiple non-classical mouse α chains also contain this tyrosine residue.

So far, there is no consensus on the molecular mechanism underlying reverse MHC-I signaling, as the existing hypotheses rely on different domains of the complex for signal transduction or different MHC-I subclasses. MHC-I molecules are heterodimers composed of a heavy α-chain, and a light chain called beta-2 microglobulin (β_2m). Typically, the α-chain contains domains α1 and α2, both involved in peptide binding, and the immunoglobulin-like α3 domain, proximal to the membrane, which is recognized by CD8 co-receptors on the T cell (1). β_2m expression is important for the stable expression of the α-chain at the cell surface, the proper binding of the complex to the antigenic peptide for presentation and for TCR recognition (20,21). The transmembrane helix of the α-chain anchors the MHC-I complex to the membrane. The α-chain also has a short cytoplasmic tail of around 30 to 40 amino acids (Fig. 1B).

Multiple genes, grouped into MHC gene clusters, encode for heavy α-chains in mouse and human, and are divided in classical and non-classical MHC molecules. The classical MHC-I molecules (MHC-Ia) are responsible for activating $CD8^+$ T cells and correspond to α-chains that are expressed at high levels and are highly polymorphic to allow for increased variability of peptide binding sites. In humans, these correspond to the HLA-A, HLA-B and HLA-C genes and in mice, these genes are H-2K, H-2D and H-2L. The non-classical MHC-I molecules (MHC-Ib) are expressed at lower levels and are less polymorphic. They participate in activation or regulation of NK cells, among other less understood functions. In humans MHC-Ib molecules include genes HLA-E, HLA-F, HLA-G and the pseudogene HLA-H, whereas in mice it includes several genes under the H2-Q, H2-T, and H2-M regions (22–25). Interestingly, the cytoplasmic domains of some classical and non-classical α-chains in different species have a conserved tyrosine residue (9,26) (Figure 1C). As discussed in the following sections, part of the evidence for reverse MHC-I signaling is supported by the notion that this tyrosine residue can be phosphorylated and that tyrosine phosphorylation is a posttranslational modification frequently involved in signal transduction (27). Besides this, based on their structure, MHC-I molecules belong to the Immunoglobulin (Ig) superfamily, which is widely involved in cellular recognition and intercellular signaling (6,28). Moreover, the existence of open conformers, cell surface MHC molecules that are not associated with peptide and/or β_2-microglobulin (β_2m), and their ability to associate with themselves or other receptors, suggest that MHC-I molecules have more functions than only antigen presentation to T and NK cells (29). The formation of open conformers is also associated with tyrosine phosphorylation, further supporting the idea of reverse signaling by MHC-I (30).

Reverse MHC-I signal transduction in myeloid immune cells

Macrophage activity regulation by reverse MHC-I signaling

Early evidence of MHC-I reverse signaling in macrophages was already reported in 1987 (Table 1). Immunoglobulin G2 (IgG2) antibodies that targeted MHC-I, but not the corresponding $F(Ab')_2$ fragments, induced guinea pig alveolar macrophages to release reactive oxygen species (ROS) in the oxidative burst (31). This increase was not observed for IgG1 antibodies, which even reduced ROS release, whereas their $F(Ab')_2$ did not have an effect (31). This finding was explained as a consequence of bipolar bridging, an event in which an antibody binds simultaneously to an Fc-receptor via its Fc-tail and to MHC-I with its antigen-binding Fab motif on the same membrane (*cis* bridging). Therefore, this finding cannot be considered as solely MHC-I reverse signaling, but as an effect of collaboration between MHC-I and the Fc receptors for IgG2 or IgG1. Notably, although the antibody Fc-tail could also bind to an Fc receptor on a different cell (*trans* bridging), different authors claim that bipolar bridging is a monocellular event (32,33). The main evidence for this comes from experiments with mast cell mixtures where only those cells expressing both the right H-2 antigen and Fc-receptor degranulate upon antibody binding (34), and with antibody-induced degranulation of single cells isolated with a micromanipulator (35) (see also section 2.3 on mast cells). Furthermore, the bipolar bridging hypothesis relies on physicochemical models proposing that the high local concentration of proteins at the plasma membrane makes it easier for an antibody to bind to MHC-I and Fc-receptors on the same cell, rather than on two different cells (36,37). The physiological relevance of simultaneous signaling during bipolar bridging is unclear, because it is unknown when for instance a $CD8^+$ T cell or an NK cell would display ligands for both MHC-I and Fc-receptors.

Table 1 Summary MHC-I signaling in macrophages.

Cell type	MHC-I stimulation	Signaling	Cell outcome	Ref
Guinea pig alveolar macrophages	Guinea pig IgG1 and -2 antibodies	-	In combination with Fc-receptor stimulation, ↑ or ↓ release of oxygen free radicals	(31)
Mouse peritoneal macrophages	TLR ligands, bacteria, monoclonal antibodies (anti-H-2Kb; AF6-88.5)	↑ Fps, SHP-2 ↓TRAF6, NF-kB	↓ TLR response	(9)
Mouse bone marrow-derived macrophages	VSV infection, IFN-β	↑ SHP2 ↓STAT1, ISG	↓ type I IFN signaling and ↑ viral infection	(6)
Human monocyte-derived dendritic cells	Synapse with $CD8^+$ T cells or beads coated with mouse anti HLA-A2 antibodies	-	Remodelling of recycling endosomes from vesicular to tubular structures	(39)
Mouse bone marrow-derived dendritic cells	B2-microglobulin knock out mice	-	Increased production of TNF, IL-6 and IFN- β after TLR stimulation	(9)

A few decades later, a modulatory effect by MHC-I reverse signaling on the toll-like receptor (TLR) signaling cascade was described. When compared to β_2m knockout mice, it was established that normal expression of MHC-I suppresses the TLR response, including the production of tumor necrosis factor (TNF), interleukin (IL)-6 and interferon-beta (IFN-β). This TLR suppression is suggested to restrict innate inflammatory responses upon bacterial infection, to prevent excessive damage as seen during septic shock (9). However, this data should be interpreted with caution since β_2m also dimerizes with the heavy chains of other MHC-I-like surface molecules such as CD1 and the neonatal Fc-receptor (FcRn), and therefore its deletion might have effects beyond MHC-I function (20). In mouse peritoneal macrophages, this MHC-I-induced inhibition of TLR-mediated inflammatory responses was shown to be induced by interaction with naïve $CD8^+$ T cells (9). Based on experiments using MHC class I ligation with antibodies, this study proposed a mechanism in which the Src homology 2 (SH2) domain of the tyrosine kinase Fps interacts with a phosphorylated tyrosine site in the cytoplasmic domain of MHC-I, leading to Fps activation (Fig. 2) (9). TLR stimulation activates the kinase Src, which was suggested to phosphorylate the tyrosine in the cytoplasmic domain of MHC-I, thus facilitating its association with Fps (9). In turn, activated Fps associates with the phosphatase SHP-2, which then interacts with TNF receptor associated factor (TRAF) 6 thereby downregulating its ubiquitination and activity. In this way, MHC-I stimulation suppresses the MyD88-TRAF6-mediated activation of NF-κB and thus inhibits TLR downstream responses (9).

In addition to the role of MHC-I reverse signaling in dampening TLR signaling, its roles in type I interferon (IFN) signaling and antiviral infections has been investigated. Using bone marrow-derived macrophages from MHC-I-deficient mice, it was shown that MHC-I promotes replication of vesicular stomatitis virus (VSV) in macrophages independent of T cells (6). Additionally, MHC-I plays a regulatory role in type I IFN signaling, which was shown by increased phosphorylation of signal transducer and activator of transcription 1 (STAT1) and elevated interferon-stimulated gene (ISG) expression in MHC-I-deficient macrophages (6). To fulfil this function, MHC-I depends on phosphorylation of its intracellular tyrosine site, as shown by immunoprecipitation. Moreover, SHP-2 was necessary for suppression of IFN signaling by MHC-I engagement, via interaction of SHP-2 with STAT1 after VSV infection (6).

In conclusion, MHC-I seems to be involved in reverse signaling in macrophages, either alone or in collaboration with Fc-receptors, thereby affecting ROS release, TLR responses and type I IFN responses. Therefore, reverse MHC-I signaling may regulate the activity of macrophages to prevent an excessive response and damage

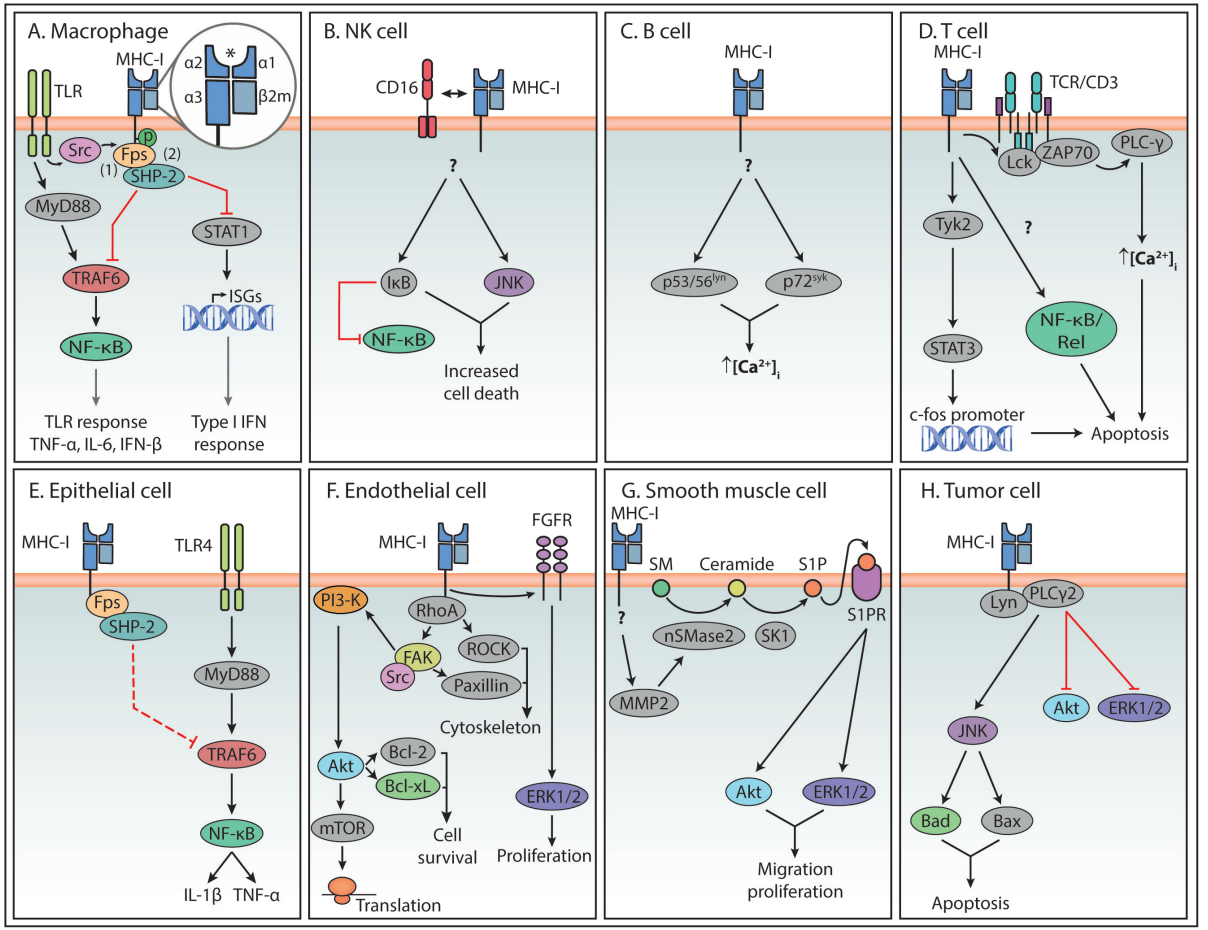

Figure 2 Summary of reverse MHC-I signaling pathways in various cell types. Summary of the main reverse MHC-I signaling pathways in immune **(A - D)** and non-immune cells **(E - H).** Question marks indicate steps in the pathway that are still unknown. Interactions represented by solid lines were confirmed to participate in reverse MHC-I signaling, while the interaction represented with a dashed line in **(E)** represents the authors' speculation. The asterisk symbol (*) indicates the antigen binding site of MHC-I. In the case of Endothelial cells **(F)**, reverse MHC-I signaling has been well studied and only a brief overview is presented here. A more detailed description can be found in a review by Valenzuela and Reed, 2011 (5).

(9). This effect could be induced after interaction with the TCR on $CD8^+$ T cells (9), but potentially also receptors on NK-cells, such as KIR receptors or C-type lectin-like receptors like Ly49 (38).

Reverse MHC-I signaling modulates membrane trafficking and cytokine production in dendritic cells

In comparison to the other cell types discussed in this review, reverse MHC-I signaling has been understudied in DCs (Table 1). In human monocyte-derived DCs (moDCs) it was demonstrated that establishment of antigen-specific immunological synapses with $CD8^+$ T cells induces the remodelling of endosomal recycling compartments (ERC), going from a vesicular to a tubular morphology (39). To dissect the molecular mechanisms of ERC remodelling, moDCs were stimulated with antibody-coated beads. These experiments revealed that ERC remodelling could be triggered by beads coated with antibodies against MHC-I (HLA-A2) and/or antibodies against ICAM-I molecules. Furthermore, remodelling was more extensive in moDCs previously activated with LPS than in immature moDCs. The tubules were suggested to facilitate the transport of antigen-loaded MHC-I molecules to the immunological synapse. However, this study did not explore the signaling pathway(s) triggered by MHC-I stimulation that lead to endosomal remodelling (39).

In the same study reporting the modulation of TLR downstream signaling by reverse MHC-I signaling in macrophages (9) (see section 2.1), it was shown that bone marrow-derived DCs (BMDCs) derived from mice lacking β2-microglobulin release higher amounts of TNF, IL-6 and IFN- β than their wild-type counterparts upon TLR stimulation. These results suggest that, similar to macrophages, MHC-I might have a regulatory role on TLR signaling in DCs (9). However, this study only studied the mechanism of reverse MHC-I signaling in macrophages.

Reverse MHC-I signaling in peritoneal cells induces mast cell degranulation

In an early study, alloantibodies against MHC molecules were generated by immunizing mice of the CBA strain with spleen cells from mice of the A/JAX strain, which has a different alloantigen profile. When serum from the CBA immunized mice was applied to BALB/c mast cells, increase degranulation from 3% in non-treated cells to 61% in treated cells was observed (40) (Table 2). This physiological degranulation was attributed to alloantibodies against H-2 molecules, mainly from the IgG1 type (41). However, it cannot be concluded that this was solely the result of MHC-I reverse signaling, because this serum may have contained antibodies against other biomolecules, such as MHC-II, and thus these experiments should be repeated with purified monoclonal anti-MHC-I antibodies. Additional experiments showed

that degranulation was caused by antibody bipolar bridging (see section 2.1 on macrophages), which is the simultaneous binding of the antibody paratopes to H-2 molecules and of the Fc tails to Fc-receptors on the same mast cell (34,40,42). This induced degranulation of mast cells was later confirmed with monoclonal IgG2a antibodies directed against H-2 antigens (43) and polyclonal antibodies against H-2 K and H-2 D (42). However, to be able to draw conclusions about the specific role of MHC-I reverse signaling in mast cells, anti-MHC-I antibodies need to be compared with their $F(ab')_2$ fragments and irrelevant antibodies, since degranulation is known to be caused by Fc-receptor signaling (44). If reverse MHC-I signaling contributes to mast cell degranulation *in vivo*, it might be involved in the inflammatory process induced by antibodies or other inflammatory cells such as $CD8^+$ T cells.

Neutrophil activity and cell death might be affected by reverse MHC-I signaling_

As shown for guinea pig macrophages, bipolar bridging of MHC and Fc-receptors on peripheral and peritoneal guinea pig neutrophils by IgG_2 induced superoxide anion ($O_2^{\bullet-}$) release (32) (Table 2). Moreover, in human peripheral blood neutrophils, ligation of MHC-I with $F(ab')_2$ fragments of the antibody W6/32 also induced signal transduction, reflected in reduced apoptosis rate (45). Furthermore, it was shown that as neutrophils start aging in culture, their apoptotic activity increases along with a reduction of MHC-I surface expression, suggesting a reciprocal relation between both phenomena. In line with this hypothesis, the *in vitro* treatment of neutrophil cultures with granulocyte-macrophage colony-stimulating factor (GM-CSF) or cyclic adenosine monophosphate (cAMP), two molecules that delay age-related apoptosis, also blocked the downregulation of MHC-I (45). Similar to mast cell degranulation, $O_2^{\bullet-}$ release and decreased apoptosis in neutrophils by reverse MHC-I signaling might contribute to inflammation upon encountering an activated inflammatory cell or infected cell.

Table 2 Summary MHC-I signaling in peritoneal cells and neutrophils.

Cell type	MHC-I stimulation	Signaling	Cell outcome	Ref
Mouse peritoneal cells	Serum of CBA mice immunized with A/JAX spleen cells	-	↑ mast cell degranulation	(40)
Mouse peritoneal cells	Anti-H-2K and anti-H2D	-	↑ mast cell degranulation	(42)
Mouse peritoneal cells	Anti-H-2 antibodies (IgG2a)	-	↑ mast cell degranulation	(43)
Peripheral and peritoneal guinea pig neutrophils	Anti-B and anti-Ia antibodies	-	↑ superoxide anion (O_2^-) release	(32)
Human peripheral blood neutrophils	Anti-MHC-I antibodies (W6/32)	-	↓ cell death	(45)

Reverse MHC-I signal transduction in lymphocytes

Reverse MHC-I signaling affects cytotoxicity and cell death of NK cells

Early studies on the role of β_2m and reverse MHC-I signaling in NK cell activity led to contradictory findings, probably because some studies used the full fraction of human peripheral blood lymphocytes or spleen leukocytes (46–49), while another study used enriched NK cells (50). While the former studies proposed that reverse MHC-I signaling in NK cells reduces their cytotoxic activity when compared to unstimulated NK cells, the latter study found the opposite (Table 3). An alternative reason for these differences could be the use of different antibodies to stimulate MHC-I. Evidence that this indeed might be the case, comes from another early study (51). Using NK cell lines, such as NK3.3, and primary NK cells, it was shown that different anti-MHC-I antibodies have either an stimulating, inhibiting or no effect on their lytic activity against cancer cell lines (e.g. leukemic K562 and lymphoblastic MOLT-4), when compared to unstimulated or irrelevant antibody (Cenox 3.53)-stimulated cells as a control (51). This variability in effects of anti-MHC-I antibodies was also observed for the induction of the oxidative burst in macrophages (31).

More recent studies found that stimulation with anti-MHC-I antibodies inhibits cytotoxic activity of human peripheral blood NK cells and NK cell lines (52–54). Reverse MHC-I signaling was studied in the context of treatment with either anti-CD16 antibodies, which inhibited NK cell cytotoxicity, or IL-2 activation, which augmented cytotoxicity. MHC-I antibodies in combination with anti-CD16 antibodies or IL-2 treatment, further inhibited cytotoxicity of human blood NK cells against several tumor cell lines when compared to an isotype antibody control (IgG1), unstimulated cells or cells stimulated only with IL-2 or CD16 (54). Since anti-MHC-I antibodies alone did not have any effect, an interaction between MHC-I and CD16 or IL-2 receptor signaling could be needed for blocking cytotoxicity. Blocking of NF-κB with a cell permeable inhibitory peptide in CD16 antibody-treated human blood NK cells reduced cytotoxicity, suggesting a role of NF-κB in the downstream signaling pathway (55). When using the NKL cell line, derived from a human leukemia patient, cytotoxic activity against K562 cells was not affected by MHC-I ligation with the mouse monoclonal W6/32 antibody (53). However, cross-linking of MHC-I molecules by further addition of sheep-anti-mouse IgG inhibited cytotoxic activity, which was confirmed for human blood NK cells (53). Compared to ligation with only a primary monoclonal antibody, efficient cross-linking requires a secondary antibody and induces clustering and interaction between the target molecules (56), potentially leading to increased reverse MHC-I signaling. The inhibition of cytotoxicity after cross-linking was not caused by an induction of NK cell apoptosis, masking of MHC-I on the target cells, or reciprocal NK cell-NK cell killing, as indicated by similar cell viability and lysis under all conditions (53). Similarly, NKL cell cytotoxicity against the P815 mastocytoma cell line triggered

Table 3 Summary MHC-I signaling in natural killer cells.

Cell type	MHC-I stimulation	Signaling	Cell outcome	Ref
NK cell line clones and NK cell bulk populations	Anti-MHC-I antibodies (131, 164, W6/32, 404, 4E, 46)	-	↑/↓/no effect on lytic activity	(51)
Human peripheral blood NK cells	Anti- β_2m antibodies (Table 1 in article)	-	↑ lytic activity	(50)
Human peripheral blood NK cells	Anti-MHC-I antibodies (PA.2.6 HB118 IgG1)	-	In combination with CD16 stimulation, ↓ NK cell cytotoxicity ↑ cell death	(54)
Human peripheral blood NK cells and NK cell line YT	Anti-MHC-I antibodies (PA.2.6 HB118 IgG1)	↑ IkB, JNK ↓ NF-κB	In combination with CD16 stimulation, ↑ NK cell death	(55)
Human peripheral blood NK cells and NKL cell line	Anti-MHC-I antibodies (W6/32 IgG2a) and secondary antibodies (sheep anti-mouse IgG F(ab')$_2$)	-	↓ conjugation with target and ↓ cytolytic activity	(53)
Human peripheral blood NK cells and NKL cell line	Anti-MHC-I antibodies (W6/32 IgG2a)	-	↓ cytotoxicity and ↓ IFN-γ production	(52)

by anti-CD16, anti-NKp46 and anti-2B4 (but not anti-NKG2D) was inhibited as well by anti-MHC-I antibodies when compared to an isotype antibody control (IgG2a) (52). Human blood NK cell cytotoxicity was also inhibited by anti-MHC-I antibodies when triggered by CD16, NKG2D, NKp46 and 2B4, while isotype antibody control (IgG2a) had no effect (52). Additionally, the IFN-γ secretion by NKL cells and primary NK cells was inhibited with anti-MHC-I antibodies, in contrast to incubation with isotype antibody controls (IgG2a or IgG2b) (52). Thus, most studies suggest a role for reverse MHC-I signaling in the inhibition of NK cell cytotoxicity.

Besides NK cell cytotoxicity, cell death after MHC-I triggering has been investigated. Stimulation of NK cells isolated from healthy human blood mononuclear cells with anti-CD16 antibodies led to increased cell apoptosis in comparison to unstimulated cells, which was further augmented by anti-MHC-I antibodies (54). The combined stimulation of CD16 and MHC-I in NK cells also induced TNF-α secretion in positive correlation with cell death, while no effect was seen on IFN-γ secretion. These results suggest that reverse MHC-I signaling plays a role in upregulating TNF-α secretion in anti-CD16-treated NK cells (54). This was further confirmed by blocking TNF-α with antibodies, which inhibited the increase in cell death induced by CD16 and MHC-I triggering, suggesting a role for TNF-α in regulating NK cell apoptosis (54). Since the functional inhibition was observed prior to the increased cell death, they were

suggested to be successive. Since, in principle, adding antibodies against MHC-I molecules on NK cells could interfere with the inhibitory signals to other NK cells, resulting in reciprocal NK-to-NK cell killing, a coculture was made with two groups of NK cells treated separately with MHC-I antibodies or CD16 antibodies. This coculture had similar cell death levels to cells treated only with anti-CD16 antibodies, and decreased cell death compared to cells treated with both antibodies simultaneously. This experiment confirmed that there is an additive signaling effect when stimulating CD-16 and MHC-I on the same cell. (55). Moreover, addition of anti-LFA-1 antibodies also did not change NK cell death, suggesting that LFA-1 mediated cell adhesion was not involved in the reverse MHC-I signaling process (55).

In an attempt to describe the signaling cascade downstream of MHC-I, focus was placed on NF-κB. Here treatment with anti-CD16 antibodies led to induction of NF-κB activity in the nucleus, while blocking of NF-κB in CD16 antibody-treated human blood NK cells induced cell death. This protective effect through CD16 stimulation was inhibited by addition of anti-MHC-I antibodies (55). Moreover, an increased expression of the NF-κB inhibitor IkB and upregulation of the proapoptotic c-Jun N-terminal kinase (JNK) was observed when both antibodies against CD16 and MHC-I were used (55). Hence, it was proposed that reduction of CD16-mediated NF-κB activity and increased JNK underlie the increased NK cell death after anti-MHC-I ligation.

Interestingly, in resting conditions MHC-I molecules were uniformly distributed on the surface of cells from the NKL cell line, similar to the control protein CD59 (also known as protectin), and co-localized with glycolipid-enriched membrane microdomains called lipid rafts. However, MHC-I cross-linking with primary and secondary antibodies before fixation excluded these molecules from lipid rafts in a similar way to the cell-surface transferrin receptors (CD45 and CD71), which locate outside the lipid rafts and were included as controls (53). Moreover, while MHC-I molecules were present in the immunological synapse between untreated NKL cells and the leukemia cell line K562, cross-linking with primary and secondary antibodies displaced MHC-I molecules to the opposite site of the NKL cell, away from the synapse (53). MHC-I cross-linking on NKL cells was also observed to induce intracellular tyrosine phosphorylation when compared to untreated NKL cells, although the progression of the MHC signaling pathway and immediate interaction partners are topics for further investigation (53).

An important aspect of NK cell biology is that they express a wide variety of receptors that recognize MHC-I. This raises the question whether these receptors could recognize MHC-I on the same cell (*cis* binding), and how this affects the interpretation of the literature cited in this section. A few studies have shown that *cis* binding indeed occurs (57,58). In mouse NK cells, the inhibitory receptors Ly49A and Ly49C,

which typically binds to H2-$D^{b,d,k}$ molecules on other cells, can also associate with the allele H-$2D^{d}$ on the same NK cell (57,58). Although MHC-I signaling was not studied in this type of *cis* binding, it is important to highlight that Ly49A inhibitory signaling does not seem to be elicited by MHC-I molecules in *cis* (59). On the contrary, *cis* binding promotes NK cell activation as Ly49A receptors bound to MHC-I in *cis* are prevented from binding MHC-I molecules on other cells (*trans* binding) (57–60). In the case of human NK cells, *cis* binding has not been confirmed, but it has been hypothesizes to occur (61). Considering that *cis* binding is not completely well understood, it is difficult to establish to what extent the use of monoclonal antibodies against MHC-I will affect *cis* binding to NK cell receptors and its equilibrium with *trans* binding. This is mostly important in the cytotoxicity assays reviewed in this section. Furthermore, it is difficult to say how well the experimental control conditions chosen by the studies presented here (e.g. unstimulated cells, or stimulation with isotype controls) account for potential background *cis* binding between MHC-I and its receptors on the NK cell surface.

In conclusion, MHC molecules might be involved in reverse MHC-I signaling in triggered NK cells, mostly leading to decreased cytotoxicity and increased cell death. Possibly, this inhibition could be a way for cancer or infected cells to escape from NK cell killing *in vivo*. Furthermore, it might be a mechanism of other (healthy) immune cells to protect themselves from being attacked by NK cells. However, which specific natural ligands of MHC-I would establish such inhibitory interactions remains unknown. Potential candidates would be members of the Ly49 family in mouse, or the LILR family in human, which are expressed by different myeloid and lymphoid cells (Reviewed by (59)). Moreover, lymphoid cells could use KIR or CD94-NKG2 receptors to communicate and regulate NK cells. In the future, consideration should be given to MHC-I itself or its downstream signaling molecules as therapeutic targets for treatment of pathologies with involvement of NK cells, such as cancer and viral infections.

The role of reverse MHC-I signaling in B cell proliferation and cell death

Cross-linking of MHC-I on human acute lymphoblastic leukemia (ALL) B cell lines and myeloid precursor cell lines by several mouse anti-MHC-I antibodies combined with a secondary rabbit-anti-mouse Ig antibody, reduced proliferation and induced apoptotic cell death, measured as DNA fragmentation. Those primary antibodies recognized epitopes in HLA-A,-B and -C or β_2m and cross-linking with the secondary antibody was strictly required for apoptosis induction (62). Interestingly, MHC-I signaling also induced apoptosis in the context of mature peripheral blood B cells activated *in vitro* with an antibody against CD40, which is considered a cell rescue signal (62). In contrast, MHC-I did not have an effect in proliferation in B cells previously activated with the mitogenic bacteria *Staphylococcus aureus* or staphylococcal enterotoxin A (62). The authors suggest that MHC-I stimulation might lead to initial B cell activation,

as seen previously in T cells, followed by cell death, based on the observation of an initial increase in metabolic activity in the ALL KM-3 cell line and a slow start of DNA fragmentation following MHC-I stimulation (12 hours) (62) (Table 4). A different result was obtained by another study in which anti-MHC-I antibodies used on purified human tonsillar B cells prevented the onset of proliferation typically induced by *S. aureus*, indicating a role of MHC-I in inhibiting B cell activation (63). On the contrary, MHC-I stimulation had no effect on B cell proliferation induced by treatment with phorbol-12-myristate-13-acetate (PMA), a diacylglycerol analog that activates protein kinase C (PKC), suggesting that the inhibitory role of MHC-I targets signaling events preceding upregulation of PKC activity (63). The fact that inhibition of proliferation was not observed with $F(ab)_2$ fragments of one of the antibodies used (W6/32) suggests that Fc-receptors are involved or that the Fc region inhibits interaction with another cell surface protein (63).

Table 4 Summary MHC-I signaling in B cells.

Cell type	MHC-I stimulation	Signaling	Cell outcome	Ref
Cell lines KM-3, HL-60 and CD40-activated B cells	Anti-MHC-I antibodies (PA2.6, W6/32, BB7.5, BB7.7 and L368) and secondary rabbit anti-mouse Ig	-	↑ cell death and ↓ proliferation	(62)
Human tonsillar B cells	Anti-MHC-I antibodies (131, 4E and W6/32)	-	↓ proliferation caused by *Staphylococcus aureus*	(63)
Human B lymphoma cell line Solubo	Anti-human β_2m antibody from rabbit serum	-	↑ activation	(64)
Human B lymphoma cell line Loukes	Rabbit anti-human β_2m antibody	↑ protein tyrosine phosphorylation	Regulates B cell homeostasis	(65)
Human B lymphoma cell lines Solubo and Loukes	Rabbit anti-human β_2m antibody and mouse anti-human HLA-ABC	↑ protein tyrosine kinases and PI-3 kinase	↓ proliferation and ↑ apoptosis	(66)

Besides proliferation, short-term intracellular changes and signaling were observed upon MHC-I stimulation of B cells. Antibody-mediated cross-linking of MHC-I on human B lymphoma cells increased intracellular calcium levels, first derived from intracellular stores and later due to calcium influx (64). This increased intracellular calcium was positively correlated with the levels of MHC-I expressed by the cells (64). The early activation marker CD69 was upregulated upon cross-linking and MHC-I did not have a costimulatory effect on the anti-IgM-mediated activity of the cells (64). Finally, it

was suggested that MHC-I can activate intracellular second messengers itself. For instance, upon MHC-I cross-linking, the protein tyrosine kinases p53/56^{lyn} and p72^{syk} are phosphorylated in human B lymphoma cells (65). Besides protein tyrosine kinases, serine/threonine kinases might be involved in reverse MHC-I signaling, since their inhibition with the molecule H7 reduced B cell apoptosis after MHC-I cross-linking (66).

As is the case for most of the cell types presented in this review, the physiological scenario in which reverse MHC-I signaling in B cells would be relevant remains unclear. Wallén-Öhman *et al.* suggested that the observed induction of apoptosis and reduction of proliferation seen upon reverse MHC-I signaling might be important for negative selection of autoreactive B cells (62). They hypothesized that, if an autoreactive B cell would target a cell bearing an MHC-I binding receptor, such as T or NK cells, the simultaneous binding of BCR-antigen and MHC-I-ligand would lead to apoptosis (62). This hypothesis was inspired by another study in which T cell precursors recognizing CTLs underwent apoptosis (67). Here, the simultaneous binding of the precursor's TCR to an endogenous antigen on the CTL and of MHC-I to CD8 on the CTL induced the precursor's death. This effect was not seen after T cell maturation (67). However, this hypothesis has not been tested yet for B cells.

The effect of reverse MHC-I signaling on proliferation, activity and apoptosis of T cells

Early studies found anti-MHC-I antibodies to either induce or inhibit T cell proliferation (68–72)(reviewed in (8)). In these studies, the outcome of MHC-I stimulation depended on whether the anti-MHC-I antibody was used alone or in combination with other stimuli, and on the concentration of antibody used. On one hand, studies indicating an induction in proliferation only used anti-MHC-I antibodies (CR11-351, Q6/64, W6/32) or in combination with exogenous IL-2, PMA or antibodies against CD2. On the other hand, anti-MHC-I antibodies had an inhibitory effect when combined with stimuli that promote proliferation, such as the T cell activating lectin phytohemagglutinin (PHA)-P or antibodies against CD8 and CD3 (e.g. OKT3) (8,69,73–75). An antibody clone designated as 4-10 had a mitogenic effect and led to TCR downregulation in resting peripheral blood isolated T cells, when immobilized on a plate or on the surface of macrophages. However, when combined with immobilized anti-CD8 antibody it reduced CD8-elicited proliferation (68). Furthermore, 72 hour incubation of peripheral blood T cells with the anti-MHC-I antibody W6/32 immobilized on culture plates at concentrations of 0.1 µg/ml and 1 µg/ml induced T cell proliferation, tyrosine phosphorylation and increased expression of TCR/CD3 and CD28. In contrast, very low amounts of immobilized W6/32 antibody (picograms/well) inhibited TCR/CD3-elicited proliferation. Importantly, incubation of the cells with W6/32 in solution had no effect on proliferation, even at a concentration of 40 µg/ml (69).

Induction of proliferation by Qa-2 (H2-Q7), a non-classical mouse MHC-I molecule, was compared to its human homolog HLA-G within a murine background (76). For this, resting splenic T cells were isolated from wild type mice and transgenic mice recombinantly expressing HLA-G. Stimulation of both protein complexes with monoclonal antibodies led to an induction of proliferation, but only when combined with a secondary antibody for cross-linking and stimulation with PMA (76). Both Qa-2 and HLA-G localized to lipid rafts. However, whilst Qa-2 localizes via a lipid glycosylphosphatidylinositol (GPI) tail, HLA-G localizes via its transmembrane domain. The mechanism for signaling was attributed to interactions with other lipid raft-located proteins as Qa-2 has no cytoplasmic domain and HLA-G has only a short 6-amino acid cytoplasmic tail (76,77) (Table 5).

Table 5 Summary MHC-I signaling in T cells.

Cell type	MHC-I stimulation	Signaling	Cell outcome	Ref
Jurkat T cell line	Anti-MHC-I (W6/32) cross-linked by supernatant from W6/32-hybridoma cell cultures	↑ Src, Bcl-2	In the stationary growth phase ↑ apoptosis.	(84)
Cytotoxic T cell clone K14B06	Anti-MHC-I antibodies (W6/32, HP-1F7, ME-1, anti-H2Dd)	-	↓ anti-CD94 redirected target lysis	(86)
Mouse splenic T cells	Anti-HLA-G clone 87G and secondary rabbit anti-mouse IgG	-	In combination with PMA, ↑ proliferation	(76)

MHC-I is also proposed to play a role in the induction of apoptosis in T cells, because anti-MHC-I antibodies induced apoptosis in resting T cells (67), activated T cells (78), and the leukemic Jurkat T cell line (64,79–82) (reviewed in (8)). Studies focused on Jurkat cells provided a detailed description of the early signaling cascade following stimulation with biotinylated anti-β2m antibodies crosslinked with avidin. It was shown that shortly after cross-linking, MHC-I signaling induced tyrosine phosphorylation of several proteins, including the ζ-chain of the TCR/CD3 complex, ZAP-70, $p56^{lck}$ and PLC-γ1, followed by an increase of intracellular Ca^{2+} (80). Although similar to TCR/CD3 signaling, MHC-I stimulation led to inferior association of ZAP-70 with other molecules, a phosphorylated version of the TCR ζ-chain with a higher molecular weight, and weaker phosphorylation of $p56^{lck}$ (80). Furthermore, reverse MHC-I signaling was dependent on the expression of TCR/CD3 complex on the cell surface. Overall, these results suggest that MHC-I uses the TCR/CD3 complex for signaling but creates a different pattern of protein phosphorylation compared to the pattern seen after direct stimulation of TCR/CD3. The main outcome was strong inhibition of proliferation and

apoptosis induction (Figure 2D) (80,83). In a similar study, crosslinking of β2m in Jurkat cells led to activation of Tyk2, but not of the other members of the JAK family Jak1, Jak 2 or Jak3. STAT3, a substrate of Tyk2, was also phosphorylated and migrated to the nucleus where it could bind to the promoter of c-fos. Expression of a dominant negative version of STAT3 interfered with apoptotic processes associated to MHC-I stimulation (79). Another study on Jurkat cells proposed that apoptosis upon MHC-I stimulation did not follow the conventional Fas/Caspase signaling pathway but rather one that includes activation of phosphoinositide 3 kinase (PI3K) followed by JNK activation (81).

Another factor determining the outcome of reverse MHC-I singaling is the cell cycle. A study reported that the effect of MHC-I ligation on apoptosis depends on the growth phase of Jurkat cells, since in exponentially growing Jurkat cells Fas- or anisomycin (SAPK/JNK activator)-induced apoptosis is prevented by MHC-I ligation. This involves Src signaling, the upregulation of anti-apoptotic protein Bcl-2 and stabilization of the mitochondrial membrane potential (84).

A potential explanation as of why MHC-I reverse signaling can have opposite effects could be the nuclear recruitment of different homo- or heterodimers from members of the NF-κB/Rel family. Stimulation with antibodies against HLA-A,-B or -C leads to nuclear location of NF-κB1 (p105/p50) homodimers, and formation of heterodimers by either NF-κB1+RelA (p65) or NF-κB1+RelB. Since NF-κB1 homodimers lack transcriptional activation domains, they are considered to have an inhibitory function (85).

MHC-I reverse signaling also affects the T cell effector function. This was demonstrated in an *in vitro* experiment based on antibody-dependent cell-mediated cytotoxicity (ADCC) (86). To achieve ADCC cancer cells were coated with antibodies against the CD94/NKG2 receptor. Because the antibodies' Fc tails bind to the Fc-receptors on the cancer cell, the paratopes of the antibody were outwardly displayed. The antibody-coated cancer cells were then mixed with $CD8^+$ cytotoxic T cells, leading to CD94-mediated T cell activation and increased CD25 expression. As a result of this interaction, the T cells were prompted to kill the cancer cells (86). This ADCC triggered by CD94 ligation was shown to be independent of TCR stimulation and could be reduced by co-ligation of MHC-I molecules on the T cell. MHC-I stimulation, however, had no effect on the elevated CD25 expression, indicating that reverse MHC-I signaling affects only one branch of the CD94-elicited signaling cascade (86). Another hallmark of CD94-mediated ADCC is the reorientation of the microtubule organizing center (MTOC) of the T cell towards the synapse with the cancer cell, the same way it happens upon CD3 stimulation. MHC-I co-ligation significantly reduced CD94-mediated MTOC reorientation, explaining its inhibitory effect on target lysis (86).

MHC-I stimulation did not affect cytotoxicity after CD3-mediated activation, and only had a partial effect after exposure to the T cell activating lectin phytohemagglutinin (PHA), confirming the existence of several signaling cascades underlying T cell activation and therefore different degrees of crosstalk with reverse MHC-I signaling (86).

MHC molecules can also interact with cytokine receptors at the plasma membrane. For instance, MHC-I can cluster with IL-9 receptor α (IL-9Rα) and IL-2 receptor (IL-2R) in lipid rafts on human T lymphoma cells (87). Although the exact effect of MHC-I on the clustering remains unknown, several anti-MHC-I antibodies (CR11-351, Q6/64 and W6/32) block the expression of the IL-2R, as well as inhibit IL-2 secretion of T cells (74,75,88). Interactions within the receptor clusters may affect the signaling capability of these receptors, and although this has not yet been proven, a similar mechanism might also explain the increased insulin receptor signaling observed when MHC-I interacted with the insulin receptor on a B-lymphoblast cell line (87,89).

Interestingly, it was observed that upon human peripheral blood lymphocyte stimulation with the T cell activating lectin PHA, the fraction of MHC-I misfolded proteins increased, as shown by immunoprecipitation with antibodies against misfolded MHC-I. The levels of misfolded MHC-I molecules peaked at the same time as T cell proliferation (30). The fact that misfolded molecules were fully glycosylated suggested that they derived from fully mature MHC-I molecules that reach the plasma membrane but lost the peptide antigen and/or the β_2m subunit. It was also found that misfolded MHC-I molecules were more likely to dimerize and presented phosphorylation of the tyrosine in their cytoplasmic tail, associated with activity of the Src-kinase Lck (30). However, it is unclear whether phosphorylation is a cause or a result of the misfolding. The study also suggested that misfolded MHC-I molecules can associate with other proteins on the cell surface, such as CD8αβ and calreticulin, potentially regulating events related to T cell activation and its intensity. The reason for these interactions might be that misfolding and the lack of peptide unmasks amino acid motifs that are hidden in the folded form, especially in the α1 and α2 domains (29,30).

Overall, reverse MHC-I signaling has been shown to be involved in modulation of the immune response by regulating proliferation, activity and apoptosis of T cells. These contradictory outcomes might be caused by the use of different T cells and varying stimuli across the different studies. Interestingly, there is even contradictory evidence on which MHC-I domain ensures reverse signal transduction, as some studies state that the cytoplasmic and transmembrane domains are not needed for reverse MHC-I signal transduction (90,91), while others propose that the transmembrane region is required, as it might facilitate interaction with other surface receptors (92). The significance of the modulation of T cell function by reverse MHC-I signaling *in vivo* is not yet clear.

It is thought that reduced cytotoxicity might play a role in the protection of target cells, which could be beneficial to prevent microbial dissemination from dying cells (86), but also disadvantageous if tumor cells are spared by this reduced cytotoxicity. Furthermore, it might prevent non-specific lysis of healthy cells. It is yet unknown which natural membrane-bound ligands of MHC-I are able to exert the proposed effects and if these effects are also present *in vivo*. Possibly, T cells can mutually activate each other via reverse MHC-I signaling, since a common ligand for MHC-I is the TCR.

Non-immune cells contribute to inflammation by reverse MHC-I signal transduction

Epithelial cell proliferation and activity upon reverse MHC-I signaling

Multiple studies have shown that the development of anti-HLA antibodies by recipients of lung transplantation plays an important role in the pathogenesis of bronchiolitis obliterans syndrome (BOS). This seems to be caused by activation of airway epithelial cells by such HLA-A antibodies (93). As shown by an *in vitro* experiment modelling the transplantation environment, binding of anti-MHC-I antibody W6/32 to the KCC-266 airway epithelial cell line induced cell proliferation within 24 hours, followed by apoptosis. The observed proliferation was accompanied by production of the growth factors platelet-derived growth factor (PDGF), heparin-binding EGF-like growth factor (HB-EGF), insulin-like growth factor 1 (IGF-1), and basic fibroblast growth factor (bFGF). These factors led to proliferation of the fibroblast cell line MRC-5, explaining the processes of tissue remodelling and formation of fibrous tissue observed in BOS (93). In a similar study, cells from the A549 lung epithelial carcinoma cell were treated with anti-HLA serum from transplantation patients who developed BOS or with the antibody W6/32. Both treatments led to induced proliferation as well as tyrosine phosphorylation of intracellular proteins. These results could be attributed to reverse signaling by MHC-I but also to signaling by MHC-II, since anti-MHC-II antibodies could be present in the serum (94) (Table 6).

Similar to macrophages, MHC-I was proposed to modulate TLR signaling in epithelial cells. *In vitro* experiments showed that miRNA or siRNA knock down of MHC-I in primary bovine endometrial epithelial cells (bEECs) and a bovine endometrial cell line (BEND), led to increased secretion of the pro-inflammatory cytokines IL-1β and TNF-α after LPS stimulation (95). In accordance, MHC-I cross-linking by antibodies on bEECs decreased production of the same cytokines. Additionally, MHC-I heavy chain-silenced bEECs and BEND cells showed increased TLR4 expression, as well as increased activation of TLR4 downstream molecules, such as myeloid differentiation primary response 88 (MyD88), TRAF6 and NF-κB. This was even confirmed by reduced NF-κB activity after MHC-I cross-linking (95). Interestingly, MHC-I cross-linking enhanced the

interaction between MHC-I and the tyrosine kinase Fps, while Fps knock down resulted in reduced levels of SHP-2 and increased cytokine production (95). This indicates that MHC-I on endometrial cells negatively regulates the TLR4-induced inflammatory response through enhancement of the Fps-SHP-2 pathway, which might be relevant in the maternal-fetal interface (Figure 2E). This signaling mechanism closely resembles the one described in macrophages in section 2.1 (9)

Altogether, MHC-I stimulation was suggested to cause proliferation and inhibit TLR-4 signaling in lung and endometrium epithelial cells, which could have different implications. In the lung, reverse MHC-I signaling might play a role in the development of bronchiolitis obliterans syndrome after lung transplantation. While in the endometrium, reverse MHC-I signaling might be involved in interactions between mother and fetus or even control congenital inflammatory reactions (95,96).

Table 6 Summary MHC-I signaling in epithelial and endothelial cells.

Cell type	MHC-I stimulation	Signaling	Cell outcome	Ref
Airway epithelial cell line KCC-266	Anti-MHC-I antibodies (W6/32)	-	↑ proliferation, growth factor production and apoptosis	(93)
A549 lung epithelial carcinoma cell line	Anti-HLA serum or W6/32-	Protein tyrosine phosphorylation	↑ proliferation	(94)
Primary bovine endometrial cells and bovine endometrial cell line	W6/32	↑ Fps, SHP-2 ↓ MyD88, TRAF6 and NF-κB t	In combination with LPS stimulation, ↓ TLR-4 signaling	(95)
Primary human aortic endothelial cells	MHC-I antibodies (IgG1 and IgG2) with a variable region of murine W6/32 and human constant region	-	↑ monocyte adhesion	(117)
Primary human aortic endothelial cells	Chimeric mouse/human pan MHC-I (IgG1), anti-HLA-A (IgG1) and human allosera	-	Complement increased MHC-I-induced monocyte adhesion	(118)
Primary human aortic endothelial cells	Anti-MHC-I antibodies W6/32 and MEM-147	↑ mTOR, RhoA, ROCK, ERM	Regulated monocyte adhesion	(116)
Primary human aortic endothelial cells	Anti-MHC-I antibody W6/32	integrin β4, YAP	Increased expression of CTGF and Cyr61 → cell proliferation and migration	(115)

Reverse MHC-I signaling in endothelial cells contributes to adverse effects after transplantation

The endothelium is a specialized form of epithelium and will therefore be separately discussed here. The role of reverse MHC-I signaling in endothelial cells has been reviewed extensively in the context of transplant vasculopathy and concomitant tissue rejection. Specifically, alloantibodies generated by the transplant recipient against MHC-I and MHC-II alloantigens present in the transplanted tissue have been linked to endothelial cell proliferation, survival and migration that can be accompanied by recruitment of inflammatory cells into the transplanted tissue (5,7,10,97,98). The organ recipient develops antibodies not only against MHC-I, but also against different types of alloantigens. However, endothelial cell lines stimulated with allosera from a rat cardiac allograft model characterized by vasculopathy and rejection *in vitro*, showed that anti-MHC-I and II antibodies were more toxic for endothelial cells, than antibodies against non-MHC antigens. This toxicity was due to complement activation upon MHC stimulation (99,100).

In contrast to most other cell types in this review, reverse MHC-I signaling in endothelial cells has been studied in more detail and is therefore better understood. Phosphorylation of multiple signaling proteins has been observed after antibody-induced MHC-I cross-linking on endothelial cells. One of the earliest events is Ras homolog (Rho) activation immediately followed by Src, focal adhesion kinase (FAK) and paxillin activation, which together contribute to cytoskeletal remodelling and formation of focal adhesions and stress fibers (101–104). Furthermore, activation of PI3K leads to the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which promotes Akt activation and its interaction with the mTOR pathway, which overall supports protein synthesis, cell survival and migration (102,103,105–109). Akt and mTOR activation can also promote cell survival by favouring the expression of the antiapoptotic molecules Bcl-xL and Bcl-2 (108,110), as well as by the inhibition of the proapoptotic protein Bad (109)(Reviewed in (5,7,10,106)). Notably, the effects of anti-MHC antibodies on endothelial cell signaling are independent of their Fc regions (108). Additionally, MHC-I stimulation can increase the sensitivity of the cell to growth factors by mobilizing their receptors to the plasma membrane. This is the case for the fibroblast growth factor receptor (FGFR), which promotes proliferation via the extracellular signal-regulated kinase (ERK) pathway (111), in a signaling cascade that is independent from one in which ERK is directly activated by MHC-I (112).

Interestingly, it was found that integrin β4 is an important interaction partner of MHC-I, as they coimmunoprecipitate when MHC-I is crosslinked. This interaction is likely mediated by the cytoplasmic domain of the MHC-I α chain, as shown by the lack of reverse signaling upon heterologous expression of α chains lacking the cytoplasmic

domain. Knocking down integrin β4 led to reduced phosphorylation of FAK, Src, Akt and ERK after MHC-I stimulation. As a consequence, endothelial cell proliferation was less when MHC-I was stimulated in the absence of integrin β4. Similarly, MHC-I α chain knock-down affected the ability of integrin β4 to induce migration (113,114). A recent study found that Src signaling under MHC-I/integrin β4 leads to translocation of the transcriptional coactivator yes associated protein (YAP) from the cytoplasm to the nucleus. Once in the nucleus, YAP enhances the expression of connective tissue growth factor (CTGF) and cysteine-rich angiogenic inducer 61 (Cyr61), which promote endothelial cell proliferation and migration (115).

Recent studies support reverse MHC-I signaling in endothelial cells, with special focus on how it affects the recruitment and adhesion of the recipient's monocytes. This is important because intragraft macrophages correlate with worse transplant outcomes (116–118) (Table 6). An *in vitro* study showed that ligation of MHC-I by antibodies for 15 minutes on primary human aortic endothelial cells was sufficient to stimulate the exocytosis of the cell adhesion molecule P-selectin. The display of P-selectin on endothelial cell monolayers facilitated the adhesion of two monocyte cell lines (U937 and MM6) via the P-selectin glycoprotein ligand-1 (PSGL-1) on their surface. Additionally, it was found that MHC-I antibodies bound to endothelial cells further favoured cell-cell adhesion by binding to Fc-receptors on the monocytes with their Fc-region (117,119). Adhesion of monocytes to anti-MHC-I antibody-treated endothelial cells was enhanced by intact complement present in the culture media compared to heat-inactivated complement. In addition, complement further promoted MHC-I-induced P-selectin expression, which was important for adhesion (118). Human IgG1 appeared to be more effective in stimulating adhesion when compared to human IgG2, in accordance with its higher affinity for Fc-receptors (117). The integrin Mac-1 (CD11b) contributed to the increase in monocyte adhesion, since neutralizing antibodies reduced adhesion (118). Importantly, there was variation between endothelial cells and monocytes from different donors, which should be considered when designing a treatment strategy for patients with transplant rejection. The *in vivo* relevance was demonstrated by an increased macrophage burden in mice with murine cardiac allografts treated with MHC-I antibodies, whereas P-selectin blockers reduced this macrophage burden (120).

Inhibition of mTOR by drugs or siRNA in endothelial cells reduced MHC-I-induced adherence of MM6 macrophage-like cells or peripheral blood mononuclear cell (PBMC)-derived monocytes (116). This reduced firm adhesion of monocytes to endothelial cells by mTOR inhibition was comparable to ICAM-1 neutralizing antibodies, suggesting that mTOR plays a role in regulating firm adhesion of monocytes to ICAM-1 on endothelial cells stimulated with MHC-I antibodies (116). Indeed, MHC-I

ligation triggers ICAM-1 clustering via mTOR and ERM signaling (116). Moreover, mTOR activation after reverse MHC-I signaling was proposed to lead to activation of RhoA, ROCK and ezrin/radixin/moesin (ERM), as investigated with the help of small molecule inhibitors (116). Mice with a heart transplant and anti-MHC-I antibody treatment showed endothelial cell swelling and intravascular activated immune cells, which was reduced by mTOR blockade (116). Using these mice, it was also observed that anti-MHC-I antibodies increased endothelial phosphorylation of ERM, which was abolished by mTOR inhibitors (116).

In summary, reverse MHC-I signaling in endothelial cells has been linked to several cell outcomes that underlie tissue rejection, such as proliferation, migration and adhesion of immune cells like monocytes. Also, multiple mediators, such as Akt, mTOR and ERK1/2, have been associated with this signaling. Understanding these signaling pathways could facilitate the development of pharmacological interventions to treat chronic antibody-dependent tissue rejection (121). Moreover, this knowledge is not only interesting in the field of transplantation outcomes, but might also be relevant for other immune responses that require trans-endothelial migration, or diseases such as atherosclerosis in which endothelial activation and immune infiltration are involved (122).

Proliferation and migration of smooth muscle cells after reverse MHC-I signaling

Reverse MHC-I signaling in smooth muscle cells has been studied in the same context of transplantation as endothelial cells (Table 7). Cross-linking with anti-MHC-I and a secondary antibody, but also with $F(ab')_2$ fragments, induced proliferation of human aortic smooth muscle cells (SMC) (123,124). The same was observed with human mesenteric SMC and a human aortic SMC cell line triggered by MHC-I ligation with the monoclonal antibodies W6/32 and MEM-147 (125,126). Moreover, proliferation was inhibited by anti-bFGF antibodies, suggesting that MHC-I stimulation triggers bFGF synthesis or uptake (123). Additionally, anti-MHC-I antibodies lead to a rapid release of stored fibroblast growth factor receptor (FGFR) to the plasma membrane and thereafter increased synthesis of FGFR (123,127). The combination of anti-MHC-I antibody W6/32 and either TNF-α or IFN-γ, increased the FGFR expression even more (127). This could be explained by the finding that both inflammatory cytokines increased the expression of MHC-I (127). Besides smooth muscle cell proliferation, migration upon MHC-I ligation has also been investigated, since both cells are involved in intimal thickening of vessels during chronic rejection of a transplant. Antibody cross-linking of MHC-I on human aortic smooth muscle cells induced wound healing. This wound healing involved proliferation and migration, but also migration alone when proliferation was inhibited by mitomycin C (124). MHC-I knockdown inhibited anti-MHC-I-mediated SMC proliferation and migration (124).

Table 7 Summary MHC-I signaling in smooth muscle cells and myeloma cells.

Cell type	MHC-I stimulation	Signaling	Cell outcome	Ref
Human aortic smooth muscle cells	Anti-MHC-I antibodies (W6/32) and goat anti-mouse IgG	Protein tyrosine phosphorylation	↑ proliferation	(123)
Human aortic smooth muscle cells	Anti-HLA-A1 IgG and W6/32	-	↑ FGFR expression	(127)
Human aortic smooth muscle cells	Anti-MHC-I (W6/32, human anti-HLA- A24/ A32 and murine anti-HLA-A2)	↑ FAK, Akt and ERK1/2	↑ proliferation and migration	(124)
Human mesenteric artery smooth muscle cells, human aortic SMC CRL-1999 cell line and murine aortic SMC CRL-2797	Anti-MHC-I (W6/32, anti-H-2D^b)	↑ MT1-MMP, MMP2 and neutral sphingomyelinase-2	↑ proliferation	(125)
Human mesenteric artery smooth muscle cells (primary and immortalized) and human aortic SMC CRL-1999 cell line	Anti-MHC-I (W6/32, MEM-147)	↑ SK1, S1P, and S1PR1/R3 signaling	↑ proliferation	(126)
Myeloma cells	Mouse anti-human β_2m (clone B2, IgG1)	↑ Lyn, PLCγ2, JNK ↓ PI3K, ERK	↑ apoptosis	(129)

At the signaling level, MHC-I ligation increased the tyrosine phosphorylation of several proteins (123,124), such as those involved in cell survival and proliferation like FAK, Akt and ERK1/2, while downregulation of MHC-I with siRNA abrogated MHC-I-induced phosphorylation (124). Additionally, decreased FAK and p-FAK after siRNA knockdown of FAK reduced the MHC-I-mediated phosphorylation of Akt and ERK1/2, indicating that their phosphorylation depends on FAK. In accordance, integrity of the actin cytoskeleton, in which FAK is involved, is important for the MHC-I-induced activation of SMC, since the phosphorylation of cellular proteins was attenuated by disruption of the cytoskeleton upon stimulation with cytochalasin D or latrunculin A (124). Overall, FAK was found to play a role in the MHC-I-mediated proliferation and migration of SMC (124).

Another major signaling pathway linked to reverse MHC-I signaling is the sphingolipid signaling pathway, related to transduction of stress signals. Early players of this pathway, such as the matrix metalloproteinase 2 (MMP2) and its target neutral sphingomyelinase-2 (nSMase2), which catalyses the hydrolysis of sphingomyelin into

ceramide, were shown to be involved in the induction of proliferation and migration by MHC-I stimulation, indicating that anti-MHC-I antibodies behave like stress-inducing agents (125). A later player of the pathway, sphingosine kinase 1 (SK1), which phosphorylates sphingosine to sphingosine-1-phosphate (S1P), and the SP1 receptors S1PR1/R3 were also required for the induction of proliferation and migration by MHC-I stimulation, as observed when using several antibodies, inhibitors and silencing by siRNA (126). Altogether, it can be proposed that after MHC-I stimulation, MMP2 is activated and acts on nSMase2 to stimulate ceramide generation (97). Ceramide can then be converted to S1P, which activates Akt and MAPK/ERK pathways and thereby promotes proliferation (97,126). However, the signaling cascades are probably more complex than this proposed model, as for instance S1P was suggested to have a role as extracellular mediator (126).

Immunodeficient SCID/beige mice grafted with human mesenteric segments and injected with W6/32 anti-MHC-I antibody showed vascular abnormalities (vasculopathy) in the transplant, which was characterized by intimal hyperplasia. These vascular abnormalities were not observed in untreated mice or mice treated with irrelevant antibody (125,126). This suggests that also *in vivo* reverse MHC-I signaling plays a role in smooth muscle cell proliferation and migration, of which the former was confirmed by an increase in proliferating cell nuclear antigen (PCNA) (125,126). Moreover, increased intimal MMP2 expression was observed, while MMP and nSMase2 inhibitors (partly) prevented the increased PCNA expression and intimal thickening (125). Besides, PCNA labelling and intimal thickening were reduced by anti-S1P antibodies, supporting the notion that W6/32 stimulates SMC proliferation via S1P. However, it is uncertain if these outcomes are caused by direct reverse MHC-I signaling or are the result of inflammatory cytokine production (126).

In conclusion, MHC-I signaling can induce SMC proliferation and migration, but the complete molecular pathway remains to be elucidated. The induction of proliferation and migration of smooth muscle cells by MHC-I-stimulation might contribute to transplant vasculopathy, a hallmark of chronic rejection after transplantation (123,124). Several reviews have addressed the role of MHC-I antibodies in SMC activation, also in relation to transplant outcomes (7,97,98,128). However, anti-MHC-I antibody-specific effects need to be validated in human cardiac transplants showing vasculopathy.

Reverse MHC-I signal transduction results in cancer cell apoptosis

The potential of β_2m stimulation in inducing myeloma cell death

To study the potential of monoclonal antibodies in cancer treatment, the effect of anti-β_2m antibodies on myeloma cells was first investigated *in vitro*. Anti-β_2m antibodies, but surprisingly not the antibody W6/32 which targets a conformational epitope in the

MHC-I α-chain, induced apoptosis of human myeloma cell lines and human primary myeloma cells. This apoptotic effect was not counteracted by myeloma pro-survival cytokines such as IL-6 and insulin-like growth factor-I (129). In contrast, non-cancer cells such as PBMCs, were not killed, which could be due to the higher expression of β_2m and HLA-ABC on myeloma cells (129). Knockdown of β_2m expression on myeloma cells by siRNA induced resistance to the apoptosis (129). Upon anti-β_2m treatment, MHC-I was internalized and caspase-9, -3, -7 and poly (ADP-ribose) polymerase (PARP) were activated in myeloma cells (129). At the same time, the proapoptotic proteins Bad and Bax were upregulated while expression levels of antiapoptotic Bcl-2 and Bcl-xL did not change (129). In combination with an increase in cytosolic cytochrome c, this indicates increased permeability of mitochondria (129). Moreover, levels of phosphorylated JNK increased, phosphorylated Akt and ERK decreased and partial JNK inhibition almost completely abrogated myeloma cell apoptosis (129). After anti-β_2m treatment, MHC-I molecules were recruited to lipid rafts and disruption of lipid raft structure by methyl-β-cyclodextrin treatment abrogated apoptosis (129). Immunoprecipitation showed that Lyn and PLCγ2 associated with MHC-I, which were both phosphorylated after antibody treatment (129). Altogether, this suggests that due to anti-β_2m antibodies, MHC-I locates to lipid rafts, activates Lyn and PLCγ2, which activate JNK and inhibit Akt and ERK pathways and finally induce myeloma cell apoptosis. In mice, anti-β_2m injections reduced tumor volumes and improved survival, whereas no other tissues were damaged, and pJNK, cleavage of caspase-9 and caspase-3 and myeloma apoptosis were induced (129). Further evidence on the potential of anti-β_2m antibodies as therapeutic agent for multiple myeloma has been reviewed extensively elsewhere (11,130).

Altogether, it is likely that β_2m is involved in the regulation of cell death in myeloma cells and it might be interesting to study the role of MHC-I (components) in other tumor cell types. Drug targeting β_2m might be a potential treatment for myeloma patients, but it should be confirmed that there are no effects on other cells than myeloma cells as suggested by Yang et al. (2006) (129). However, the ability of tumor cells to reduce their surface MHC-I expression should be taken into account (131). It could also be useful to study if and to which extent cell death of tumor cells by ligation of MHC-I or β_2m occurs in cancer patients. Finally, as also mentioned in section 2.1, the effects triggered by anti-β_2m antibodies might not be exclusively related to reverse MHC-I signaling, since other heterodimers such as CD1 and the neonatal Fc-receptor (FcRn) are also formed by β_2m. Hence, the interpretation of the data requires caution.

Concluding remarks

Here we summarized available evidence of reverse MHC-I signaling. Reverse MHC-I signaling has been observed in multiple immune and non-immune cells *in vitro* as well as *in vivo*. MHC-I signaling has been shown to affect cell apoptosis, activation (e.g. growth factor production), proliferation, cytotoxicity and migration in many cell types, including immune cells, epithelial cells and tumor cells (3,5,6). Because of these broad actions, reverse MHC-I signaling is relevant to several fields, including research into viral and bacterial infections (6,9), transplantation outcomes (9) and malignancies (11). One difficulty in the study of reverse MHC-I signaling has been separating it from the canonical roles of MHC-I in antigen presentation for T cell activation and NK cell suppression. This is technically difficult because deletion of MHC-I would interfere with both these functions and would therefore also impair the formation of the immunological synapse. As a consequence, most studies have used monoclonal antibodies to stimulate cell surface MHC-I, leaving the physiological relevance *in vivo* to be elucidated. Similarly, the *in vivo* ligands that induce reverse MHC-I signaling are often unknown. One exception is observed in transplantation biology, where anti-MHC-I antibodies are produced by the tissue recipient.

Identifying the MHC-I domain important for reverse signaling has been controversial. Wagner et al. (1994) concluded that the cytoplasmic domain of MHC-I is not needed for signal transduction, whereas the transmembrane part is (92). In contrast, others found that neither the cytoplasmic nor the transmembrane region of MHC-I are needed for reverse MHC-I signaling, and suggested that the extracellular domain interacts with other cell surface molecules to ensure signal transduction (90,91). More recently, cytoplasmic tyrosine phosphorylation of MHC-I was observed and phosphorylated MHC-I was co-immunoprecipitated with Fps, suggesting a role of the cytoplasmic domain in reverse signaling (9). Similarly, others suggest that the first event following MHC-I ligation is an interaction of MHC-I with intracellular Lyn and PLCy2 in tumor cells (129) or association with other surface receptors on T cells (90). Additionally, the presence of putative PDZ (PSD95/disc large/zonula occludens-1) ligand motifs in the cytoplasmic region of MHC-I suggests potential interactions with PDZ domain-containing proteins *in vivo* (26). This is further supported by the fact that these PDZ ligand-like regions are conserved across species and seem to be under positive selective pressure. Moreover, *in vitro* experiments showing that MHC-I cytoplasmic domains can bind to immobilized PDZ domain peptides, are the starting point to study MHC-I downstream signaling via its cytoplasmic domain (26). Nevertheless, it is possible that the cytoplasmic, transmembrane and extracellular domains of MHC-I are all important for signal transduction, depending on the cell type and stimulus.

Although the exact pathways following MHC-I ligation are not yet completely known for all cell types, several mechanisms have been suggested (Figure 2). The emerging picture shows commonalities between different cell types, where signaling molecules/ pathways triggered by reverse MHC-I signaling are shared: Fps and SHP-2 as early events in macrophages and epithelial cells (9,95); the involvement of STAT proteins in macrophages and T-cells (6,79); JNK in NK cells and tumor cells (55,129); Akt and ERK1/2 signaling in endothelial cells (103,105,107–110,112), smooth muscle cells (124,126), and myeloma cells (129); and an inhibition or activation of NF-κB in macrophages, NK cells, T cells and epithelial cells (9,55,85,95) (Table 1-8). However, as not all of these signaling pathways are studied in all cell types, it might well be that the reverse MHC-I signaling mechanism is actually quite similar in different cell types. Also, the signaling pathway and outcome might depend on the cell subtype (e.g. resting blood T cells vs. Jurkat T cells), differentiation state, type of MHC-I stimulus, and co-stimulation (45,62). A recurrent pattern among different cell types is the crosstalk between reverse MHC-I signaling and signaling pathways initiated by for instance TLR, Fc receptors, integrins and growth factor receptors (9,31,95,112–114). Such a cross-talk with other signaling pathways would position MHC-I molecules as major modulators of immunological physiology and homeostasis.

Interestingly, similar outcomes and signaling pathways to MHC-I stimulation have been described after stimulation of MHC-II molecules in professional antigen-presenting cells, such as B cells and DCs (reviewed in (132), as well as in other immune cells, like T cells and monocytes (133–135). In B cells, reverse MHC-II signaling was suggested to play a role in B cell activation, proliferation, differentiation or apoptosis after B cell-T cell interaction (reviewed in (136–138)), and also killing of malignant B cells (136,139). Similar to MHC-I, both cytoplasmic tail-dependent and –independent mechanisms have been proposed for reversed MHC-II signaling (136,137). Another similarity to reverse MHC-I signaling is the location of MHC-II signaling molecules in lipid rafts. While stimulated MHC-II molecules present in lipid rafts of antigen-presenting cells can signal via tyrosine kinases to ensure activation, maturation or proliferation, the MHC-II molecules that are relocated to non-raft regions can signal via protein kinase C to induce cell death (132). Likewise, in murine B cells the way in which the α- and β- chains of MHC-II interact with each other creates different conformers, 10% of which locate preferentially in lipid rafts where they interact with the B cell receptor (BCR) partner CD79 to induce tyrosine kinase activity (140). Other intracellular reverse MHC-II signaling events similar to reverse MHC-I signaling are for example tyrosine phosphorylation and activation of Src, AKT, ERK and JNK (136). Also, the transmembrane adaptor protein SCIMP was shown to be tyrosine phosphorylated after MHC-II stimulation serving as scaffold for downstream signaling pathways (141). A potential role of SCIMP or a similar scaffold protein in MHC-I signaling could explain

the connection observed to tyrosine kinases. T cell studies showed that similar to MHC-I, MHC-II ligation also inhibits the activation cascades elicited by CD3 stimulation with OKT3. This inhibition was evidenced by reduced proliferation and reduced expression of the cytokines IL-1β, IL-6 and IL-2, and of the IL-2 receptor when compared to T cells treated with only OKT3 (133–135). While reverse MHC-I signaling has been poorly studied in DCs, studies on MHC-II in these cells showed induced maturation and apoptosis. Monocyte-derived DCs underwent maturation when stimulated with a pan-HLA-DR epitope (PADRE) when bound to particles, in a process involving MAP kinase, $p72^{syk}$ and NF-κB (142). Moreover, stimulation of HLA-DR molecules in plasmacytoid DCs lead to apoptosis but only when already mature (143). These commonalities strengthen the idea of a general regulatory role for MHC molecules on immune and non-immune cells, beyond their antigen presenting function.

Looking forward, the field of reverse MHC-I signaling has great potential for basic and applied research. Despite that it is increasingly clear that reverse MHC-I singaling has major effects on many cell types, our knowledge of this process is still in its infancy. The main limitation in the field is our poor knowledge of the ligands that bind MHC-I *in vivo*. Potential candidates include the members of the human KIR family and their murine functional homologs in the Ly49 family, members of the LILR and PIR family, as well as members of the CD94-NKG2 family. The fact that the expression of some of these receptors is not limited to NK and T cells, but extends to myeloid cells (reviewed in (59)), opens the possibility that multiple types of cell-cell interactions could be shaped by reverse MHC-I signaling. Moreover, the search for MHC-I ligands could be broaden to other non-immune receptors, as shown by a study on the role of MHC-I in neuronal development. This study proposes that MHC-I and the insulin receptor are expressed in different sets of neurons and can bind to each other in *trans*. This interaction allows MHC-I to downregulate the synapse-promoting role of the insulin receptor, leading to a reduced density of synapses during brain development. However, this study did not look at potential signaling downstream of MHC-I (144). Another important challenge in the field is to establish what the first signaling event after MHC-I stimulation is, as this is required for a complete understanding of reverse MHC-I signaling. In many of the studies cited in this review, this first molecular interaction was elusive and only the downstream signaling pathways were confirmed. Confirming functional relevance of the putative PDZ domains of MHC-I (26) and identifying their interaction partners (e.g. by mass spectrometry) could help to identify these early signaling events. Moreover, the reported recruitment of MHC-I molecules to lipid rafts raises the question whether reverse MHC-I signaling is regulated by the clustering and segregation of MHC-I with other receptors (53,76,77,87,129). It would also be important to study whether the signaling processes reported previously via the different MHC-I domains (cytoplasmic, transmembrane, extracellular or β2m) can actually coexist, expanding the modulatory

capacity of MHC-I. In order to address these questions, approaches used in the past for the study of TCR and BCR signaling could come in handy, using for example purified ligand candidates (as mentioned above) tethered to supported lipid bilayers (145,146). Furthermore, it would be important to assess the contribution of reverse MHC-I signaling cellular outcomes, such as proliferation and apoptosis, to *in vivo* physiological and pathological processes. Finally, future efforts could focus on the identification of potential therapeutic approaches to target reverse MHC-I singaling for managing tissue transplantation (see for example (121)), and treatment of viral and bacterial infections, as well as malignancies. If successful, this new knowledge would establish that MHC molecules are not only passive displays of antigenic peptides, but are also genuine immune receptors that modulate the intracellular signaling within the APC.

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Chapter 7

MHC class I reverse signaling triggers MTOC polarization

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Manuscript in preparation

Abstract

In response to infection, dendritic cells (DCs) have a pivotal role in the activation of both helper and cytotoxic T cells. The tight contact that facilitates the communication between these two immune cells is called the immunological synapse. To define the immune response, this contact is followed by the polarization of the microtubule organizing centers (MTOC) and secretory machineries of both cells towards the site of contact. This polarization allows the local delivery of activation signals by the DC including antigen peptides, co-stimulatory molecules and cytokines, to an antigen-specific T cell, while preventing activation of nearby non-specific T cells. While MTOC polarization is well-understood in T cells, the signals that drive polarization in DCs are unclear. Here, we used beads coated with T cell orchestrators to identify the molecules that initiate polarization of the MTOC in the DC. Our data show that MTOC and Golgi polarization are triggered via reverse signaling by major histocompatibility complex class I (MHC-I). The encountering of an antigen-recognizing T cell by MHC-I prompts signaling of Src and Syk kinases and the phospholipase C/protein kinase C signaling axis. This study shows that MHC-I is the key orchestrator of MTOC polarization which explains how DCs can specifically activate antigen-recognizing T cells in crowded lymph nodes.

Introduction

Dendritic cells (DCs) are key players in many immunological processes, ranging from destroying pathogen infected cells or cancer cells to autoimmunity. The DC is an innate immune cell that functions as the connection between innate and adaptive immunity by phagocytosis of malignant cells and pathogens which are then presented on the DC's membrane to activate T lymphocytes (1).

The activation of naive $CD8^+$ cytotoxic T lymphocytes (CTLs) requires the formation of a tight cellular interface between a dendritic cell (DC) and a T cell, called immunological synapse. Peptide fragments derived from ingested or expressed proteins are presented in major histocompatibility complex class I (MHC-I) on the surface of the DC to naive T cells in the lymphatics (2–5). This occurs via a process called repertoire scanning, where the DCs sequentially present peptides to a large number of T cells to identify T cell clones with cognate antigen-specific T cell receptors (TCRs) (6). In the absence of cognate antigen, migrating T cells only briefly interact with DCs in lymph nodes (< 3 min contact) (7). Here, only naive T cells are activated that express a cognate T cell receptor (TCR) that recognizes the peptide fragment presented in MHC-I at sufficiently high levels (7–9). Once such an antigen-specific T cell is encountered, DCs form an immunological synapse which can be stable for hours (10). The activation of naive T cells requires not only the engagement of the TCR with the MHC-peptide complex, but also co-stimulation by membrane bound receptors and soluble cytokines (2–5).

The engagement of a DC with a cognate T cell triggers a reorganization of the microtubular cytoskeleton within the cytoplasm of the DC resulting in the migration of the MTOC to the immunological synapse. This MTOC reorientation is paired with the translocation of the Golgi apparatus and the secretory machinery towards the immunological synapse in a process that depends on the Rho GTPase Cdc42 (11,12). This leads to secretion of the T cell activating cytokine Interleukin (IL)-12 at the immunological synapse that occurs from vesicles of late endosomal nature by the action of the SNARE protein VAMP7 (13). Such a reorientation of the MTOC is a common feature of immune cells engaged in synapse formation, and is also present in T cells, B cells and natural killer (NK) cells (1,14). For most of these immune cell types, the signals that trigger MTOC polarization are known (15–17). For example, MTOC polarization in B cells is triggered by the B cell receptor (18) while it is triggered in NK cells by CD28, NKG2D, NKp30 and CD94 signaling (19). In T cells, microtubule reorganization at the immunological synapse depends on TCR downstream signaling effectors, including protein tyrosine kinases Lck and Fyn (16,20,21) and ZAP70 (f-associated protein of 70

kDa) (22) and on a protein kinase C (PKC) dependent signaling cascade that induces cytoskeletal reorganization by dynein-dependent trafficking of the MTOC towards the APC (3,4,23).

However, the signals that trigger relocation of the MTOC at the immunological synapse in DCs are largely unknown. In this study, we use human and mouse synapse models between DCs and $CD8^+$ T cells to further describe the signaling mechanisms leading to T cell activation and cytokine release at the immunological synapse. Using beads coated with antibodies recognizing MHC class I, we show that polarization of the MTOC is mediated by ligation of MHC-I. This process, where MHC-I acts as a signaling receptor is called reverse MHC-I signaling (24). Our data shows that in DCs, MHC-I reverse signaling triggers tyrosine phosphorylation within the DC. Moreover, MTOC polarization could be blocked by small molecule inhibitors of Scr and Syk kinases and of PLC and PKC. Altogether, our data shows that MHC-I is the key triggering molecule at the DC synapse interface that initiates MTOC polarization, which is crucial information to understand the dynamics between T cells and DCs, leading to elimination of pathogens and malignant cells.

Results

Polarization of the Golgi network in murine DC-T cell synapses

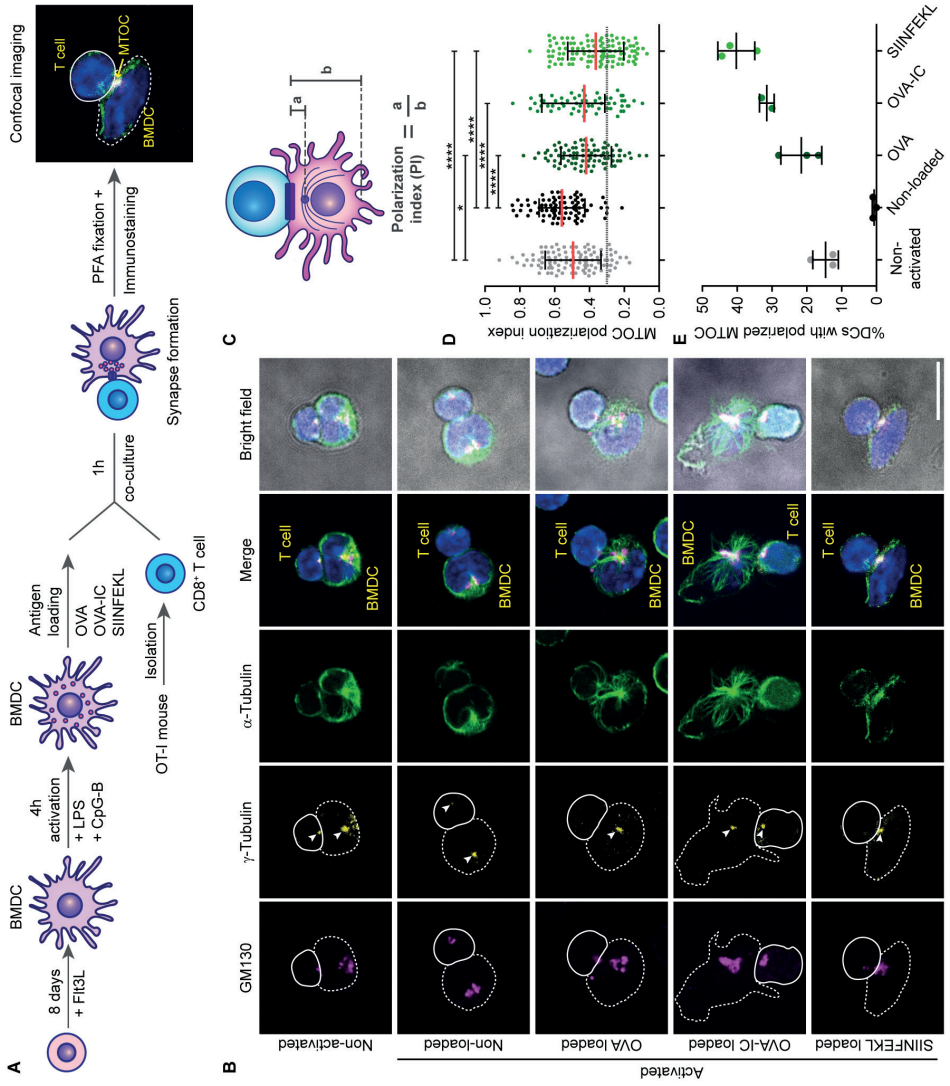
We first wanted to confirm the polarization of the MTOC in DCs towards the interface with naive CTLs and determine whether the Golgi network translocates to the DC side of the immunological synapse (12). To induce synapse formation, murine Flt3L differentiated bone-marrow derived DCs (BMDCs) were activated with ligands for Toll-like receptors (lipopolysaccharide (LPS), TLR4 agonist; CpG, TLR 9 agonist) for 4h and loaded with the model antigens ovalbumin (OVA), OVA-Immunocomplexes (OVA-IC), which consist of OVA complexed with antibodies raised against OVA, or the MHC class I epitope SIINFEKL. The BMDCs were then co-cultured for one hour with OT-I $CD8^+$ T cells that express a T cell receptor recognizing peptide SIINFEKL derived from ovalbumin (OVA) in the context of MHC-I (H-2Kb) (Fig. 1A). The cells were subsequently fixed with paraformaldehyde (PFA) and immunostained for α-tubulin and γ-tubulin, to identify the tubulin network and the MTOC respectively (Fig. 1B). Co-staining for the Golgi marker GM130 showed that the Golgi apparatus was surrounding the MTOC regardless of the activation state of the DC, the antigen or the presence of T cells.

To quantify the polarization to the immunological synapse in the DCs, the polarization index of the MTOC was calculated by measuring the distance between the MTOC and the DC-T cell interface divided by the diameter of the DC (Fig. 1C), as reported

previously (17,25). DCs with a polarization index below 0.3 were considered polarized and displayed as percentage of polarized cells as shown before (25) (Fig. 1D-E). In line with previous research (12,13), MTOC and Golgi polarization was observable in DCs that were both activated with TLR ligands and loaded with antigen. TLR activated DCs that were not loaded with antigen did not show polarization, while quantification showed that DCs loaded with OVA, OVA-IC or SIINFEKL all displayed MTOC polarisation to the synapse. Transmission electron microscopy also revealed the localization of the MTOC at the synapse in both the BMDC and the T cells for the majority of cells (Fig. 1F). In these experiments, we observed that the plasma membranes of each cell were pressed flat against each other. We did not observe membrane ruffles of pseudopodia-like protrusive structures as observed previously between CTLs and target cells (5,15). Instead, despite the short co-culturing time of the BMDCs and the CTLs (30 min), we observed a smooth membrane interface that seems more reminiscent of the late stage (>4 h) synapses observed between helper T cells and B cells and DCs (17). Thus, the Golgi apparatus is transported together with the MTOC to the immunological synapse with CTLs.

MHC-I induces MTOC polarization in DCs

The exact signals in the DC leading to the MTOC polarization are unknown. Based on the receptors triggering MTOC polarization in B, T and NK cells (18,19,23), we selected the following candidates on the DC membrane that might potentially trigger MTOC polarization: antigen presentation receptors (MHCI and MHC-II) (26,27), costimulatory molecules (CD80/86) (28) and adhesion molecules (LFA-I and ICAM-I) (12). To identify the molecule leading to MTOC polarization we developed a model where mouse Flt3L-differentiated BMDCs were stimulated with antibody or protein domain-coated beads (Fig. 2A). Non-activated BMDCs and BMDCs stimulated with naked beads or isotype control antibody were included as negative control conditions. To visualize the polarization of the MTOC and the Golgi network in our bead synapse formation model, cells were again immunostained for the α- and γ-tubulin network and GM130 (Fig. 2B). Quantification of the polarization index revealed that BMDCs only polarized when conjugated to beads coated with antibody against MHC-I and not with antibodies recognizing MHC-II, ICAM-1 or protein domains CD28 (ligand of CD80) and LFA-I (Fig. 2C-D). Also for human blood monocyte-derived DCs (moDCs), we observed polarization of the MTOC and the Golgi when stimulated with beads conjugated to MHC-I antibody and not when stimulated with naked beads or beads that were conjugated to an irrelevant antibody (isotype control) (Fig. 2E-G). Thus, MTOC and Golgi polarization during synapse formation in mouse and human DCs is induced by reverse MHC-I signaling.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Immunostaining
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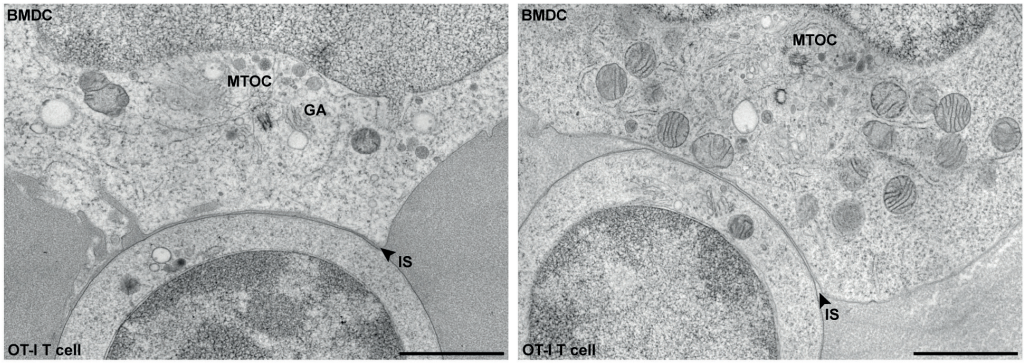
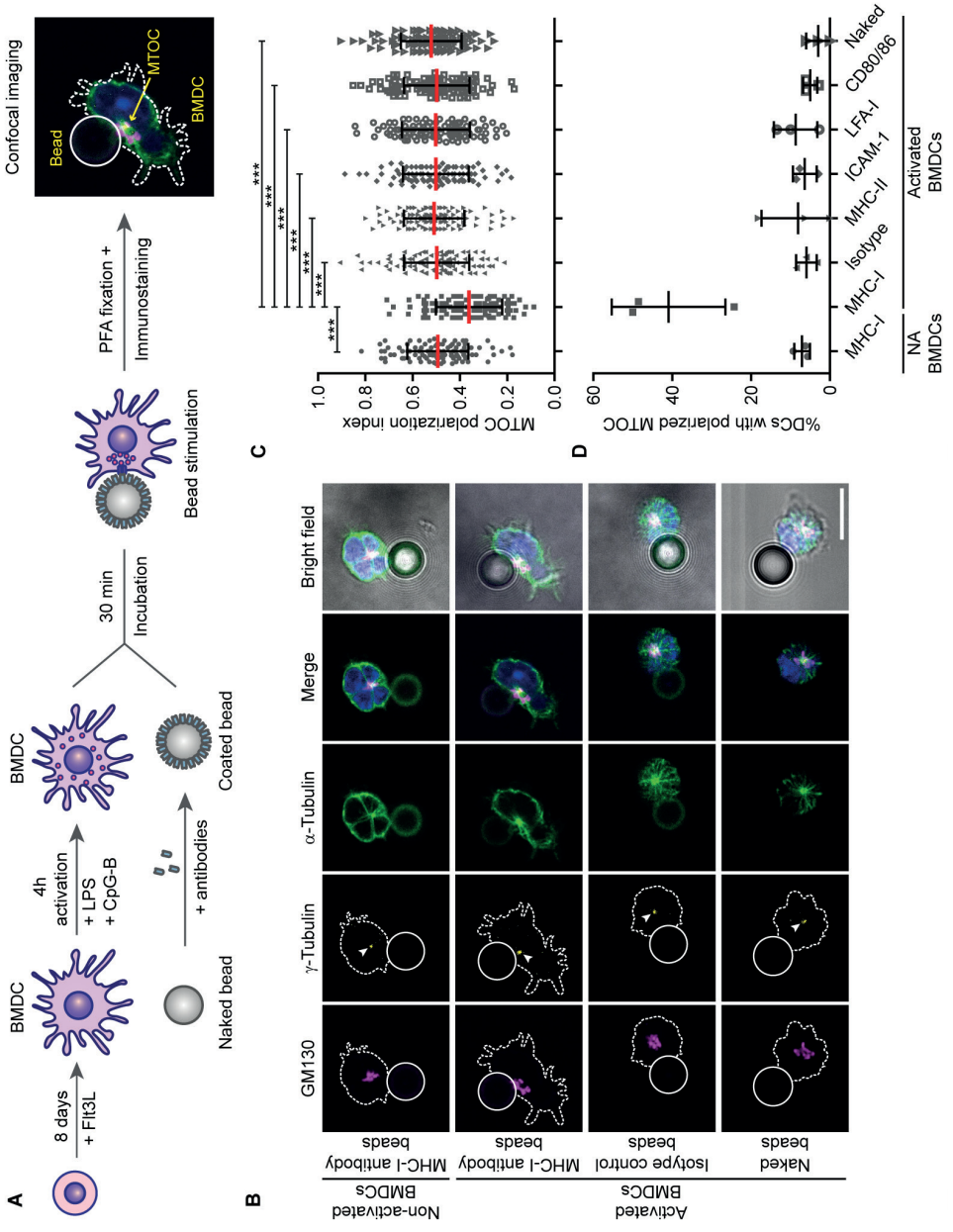


Figure 1. Immunological synapse formation triggers MTOC polarisation. (A) Scheme of immunological synapse formation between T cell and BMDC. LPS: lipopolysaccharide; CpG-B: oligodeoxynucleotide; PFA: paraformaldehyde. **(B)** Representative immunofluorescence images of the BMDC-OT-I cell synapses stained for cis-Golgi marker GM-130 (magenta), γ-tubulin (yellow), α-Tubulin (green). Yellow arrow: MTOC polarization. DAPI, blue. **(C)** Scheme of quantification of MTOC polarization: distance between the MTOC and the DC-T cell interface (a) was divided by the diameter of the DC (b). **(D)** Quantification of MTOC polarization with unstimulated BMDCs (gray), TLR ligands stimulated BMDCs unloaded (black) or loaded with OVA, OVA-Immunocomplexes or SIINFEKL (green). For each mouse culture, 30-35 cells were analysed. Mean ± standard error of the mean, Kruskal-Wallis **P<0.01, ***P<0.001. **(E)** Same as panel D, but now displayed as percentage DCs with polarized MTOC (polarization index<0.3) for individual experiments. **(F)** Representative electron micrographs of immunological synapse, showing MTOC, golgi (G), mitochondria (M) and nucleus (N).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Bead stimulation
PFA fixation +
Immunostaining
Confocal imaging
Bead
MTOC
BMDC
B
GM130
γ-Tubulin
α-Tubulin
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Bright field
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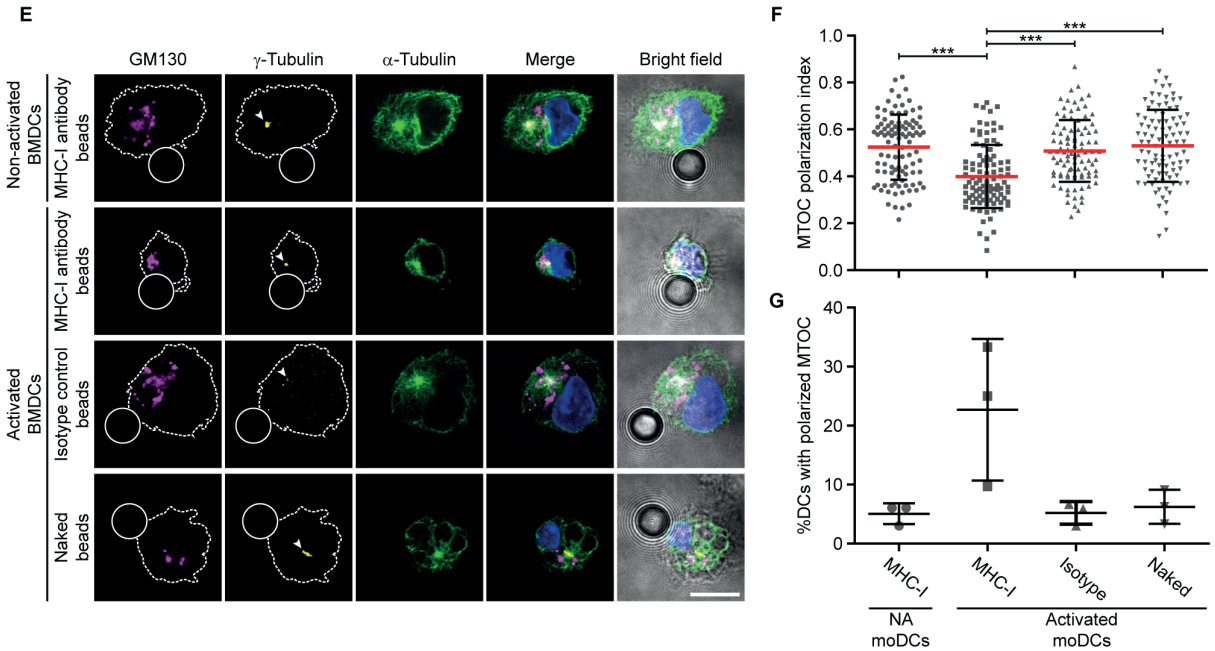
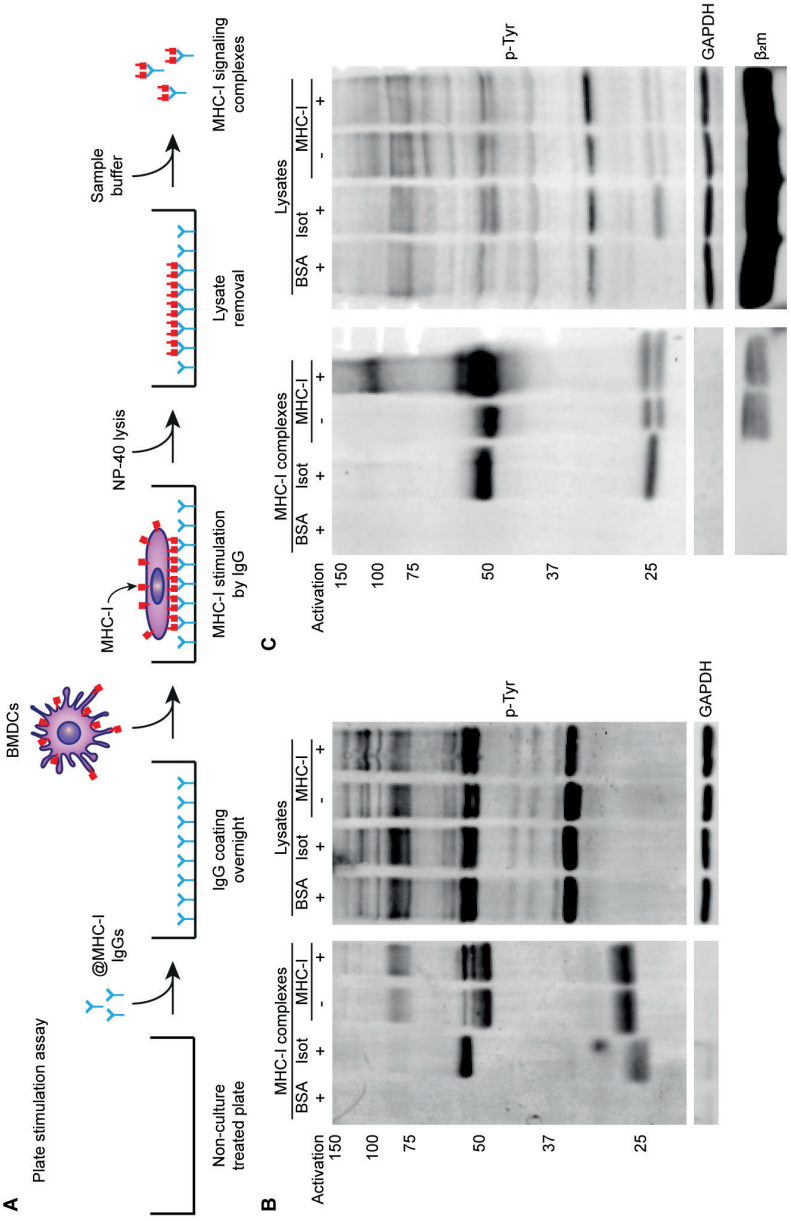


Figure 2. Beads directed against MHC-I induce MTOC polarization in BMDCs. (A) Scheme of MTOC polarization by coated-bead stimulation of Flt3L-differentiated BMDCs. LPS: lipopolysaccharide; CpG-B: oligodeoxynucleotide; PFA: paraformaldehyde. **(B)** Representative immunofluorescence images of the BMDC-bead conjugates. Beads were coated with isotype antibody or antibody against MHC-I or MHC-II. Solid outline: bead, Dotted outline: BMDC. Yellow arrow: MTOC polarization. **(C)** Quantification of MTOC polarization in BMDCs incubated with beads coated with antibodies against MHC-I, MHC-II, ICAM-1, LFA-I or CD80/86. Mean ± standard error of the mean (***P<0.001). **(D)** Same as panel C, but now displayed as percentage polarized cells for individual experiments. **(E)** Analysis of MTOC polarization in human monocyte-derived DCs (moDCs) using beads coated with isotype antibody or antibody against MHC-I. **(F)** Same as panel E, but now displayed as percentage polarized cells for individual donors.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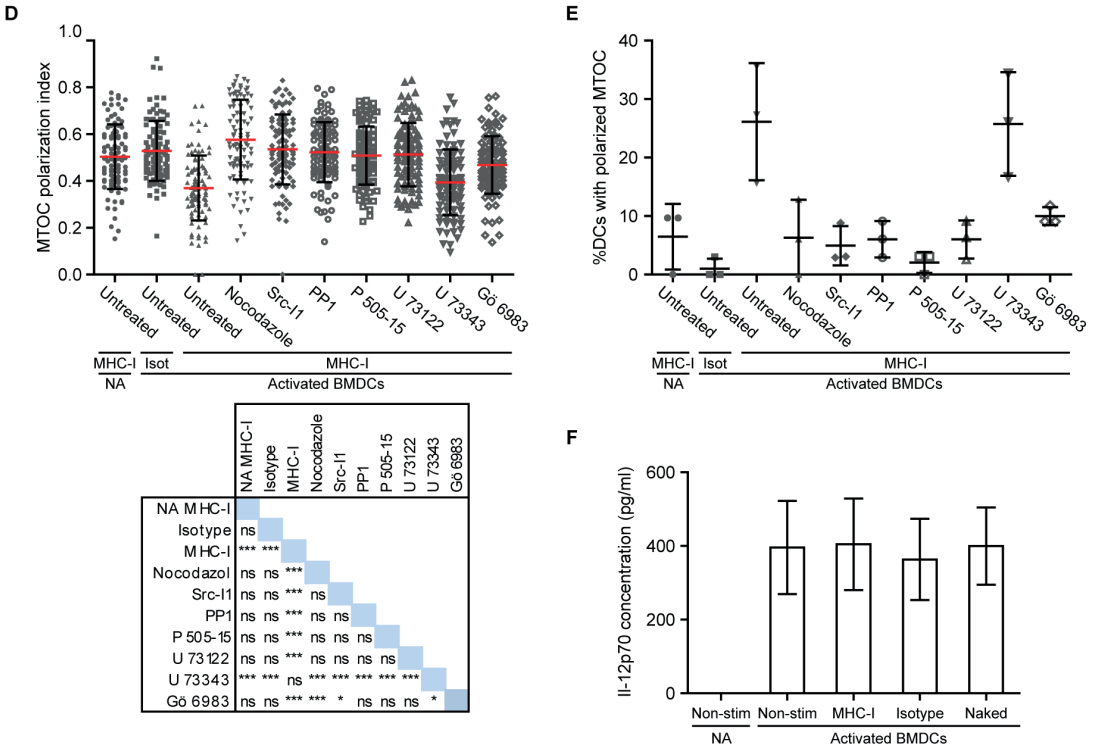

Figure 3. MHC reverse signaling triggers MTOC polarization via a signaling cascade involving Src and Syk kinases, phospholipase C (PLC) and protein kinase C (PKC). (A) Scheme of experiment to identify signaling complexes of MHC-I on plates coated with antibody recognizing MHC-I. **(B)** Western blot analysis of complexes isolated from murine BMDCs and complete cell lysates. Blots were stained with an antibody recognizing pan-phospho-tyrosines (p-Tyr) and GAPDH as loading control. **(C)** Same as panel B, but now for both inactivated and CpG-activated human monocyte-derived DCs (moDCs). β2-MG: the beta-2 microglobulin subunit of MHC-I. **(D)** Quantification of MTOC polarization in BMDCs incubated with beads coated with antibodies against MHC-I or isotype antibody control and small molecule inhibitors nocodazole (disrupts microtubules), Src-I and PP1 (inhibit Src kinase), P505 (inhibits Syk kinase), Gö6983 (inhibits PKC) and U73122 (inhibits PLC). U73343: negative control compound for U73122. Statistical significance in table. (*P<0.05; ***P<0.001; ns: not significant). **(E)** Same as panel E, but now displayed as percentage polarized cells for individual donors. **(F)** Bead conjugation does not result in altered IL-12 production.

Involvement of Src, Syk kinase and PKC signaling pathways in MTOC polarisation in DCs

In the next set of experiments, we addressed the signaling cascades downstream of MHC-I. In T cells, microtubule reorganization at the immunological synapse depends on TCR downstream signaling effectors, like the protein tyrosine kinases, including Src family of protein tyrosine kinases Lck and Fyn (16,20,21), and the Syk-family tyrosine kinase ZAP70 (f-associated protein of 70 kDa) (22). We therefore determined whether reverse MHC class I signaling triggered by antibody ligation would result in tyrosine phosphorylation. Coating of plates with a monoclonal antibody against MHC-I (Fig. 3A), showed that MHC-I stimulation leads to tyrosine phosphorylation in mouse Flt3L-differentiated BMDCs and human moDCs (Fig. 3B-C). In contrast, stimulation with only BSA or with plates coated with an isotype control antibody did not result in altered phosphorylation.

Since these experiments were in line with the involvement of tyrosine kinases in reverse MHC-I signaling, we determined MTOC polarization with MHC-I antibody coated beads in the presence of small molecule inhibitors of Src (Src-I and PP1) and Syk (P505) family kinases. Because MTOC polarization in T cells depends on signaling by PLC (phospholipase C) and several PKC (protein kinase C) isoforms (29), we also used small molecule inhibitors of PKC (Gö6983) and PLC (U73122) in our assay. As a positive control, we included Nocodazole that disrupt microtubules. After two hours of incubation with these inhibitors, the beads coated with antibody against MHC-I were added and MTOC polarization was assessed using immunofluorescence microscopy. Quantitative analysis of the polarisation index showed that all these small molecule inhibitors could block MTOC polarisation in DCs, whereas the negative control compound (U73343) resulted in normal MTOC polarization (Fig. 3D-E). In BMDCs and macrophages, reverse MHC-I signaling has also been shown to suppress cytokine production (TNF-α, IL-6) via inhibition of NF-κB signaling. However, we did not observe significantly altered production of IL-12 upon conjugation to beads coated with MHC-I antibodies compared to naked beads and antibody isotype control (Fig. 3F).

Discussion

In this study, we showed that MTOC polarization in DCs at the interface with T cells is triggered by reverse MHC-I signaling. MHC-I reverse signaling has been observed in different immune cell types, such as macrophages, NK cells, T cells, and B cells, and non-immune cell types such as endothelial and smooth muscle cells (24,30). MHC-I reverse signaling can affect a wide range of cellular processes including cell activation, proliferation, maturation, cytotoxicity, migration and apoptosis (24,30–

32). The intracellular signaling mechanism of MHC-I is not well understood, because both human and murine MHC-I forms have only a very short cytosolic motif (~30 residues) that contains no known signaling region. However, most MHC-I forms contain a conserved tyrosine that can become phosphorylated and has therefore been suggested to be involved in signaling (24). Despite this, MHC-I ligation has been shown to trigger a wide range of signaling pathways, including Fps and SHP-2 in macrophages and epithelial cells (33,34), STAT signaling in macrophages and T-cells (32,35), JNK in NK cells and tumor cells (36,37), Akt and ERK1/2 signaling in endothelial cells (38,39), smooth muscle cells (40,41), and myeloma cells (42), and inhibition of NF-κB signaling in macrophages, NK cells, T cells and epithelial cells (33,36,43). Our study now shows that, in DCs, MHC-I ligation triggers tyrosine phosphorylation. Moreover, we show that inhibition of the Src and Syk family of tyrosine kinases and of the PKC and PLC signaling axis blocks MTOC polarization downstream of MHC-I reverse signaling. Interestingly, these signaling pathways are also involved in the MTOC polarisation in T cells (16,20–22,29), where reorientation of the MTOC to the immunological synapse also facilitates membrane trafficking over microtubules to the synapse (44,45). We therefore conclude that reverse MHC-I signaling results in translocation of the MTOC to the immunological synapse in a manner dependent on Src and Syk kinases and PKC and PLC signaling.

Reverse MHC signaling likely underlies the polarized trafficking events to the immunological synapse in DCs. Such polarized trafficking has not only been described for IL-12 (11–13,46), but also for other cargo molecules including CD40 (47) and both MHC-I and MHC-II (48–51). Although trafficking of MHC-I and MHC-II do not seem to involve the translocation of the MTOC and the Golgi network, but rather the extension of tubular compartments from recycling endosomes (for MHC-I) and the MHC-II containing compartment (MIIC; for MHC-II) (48–51), evidence suggests that this DC polarization is also mediated by reverse MHC signaling. For MHC-I, tubulation of MHC-I-containing early recycling compartments in human DCs requires antigen-specific T cell interaction and involves interactions of both MHC-I and ICAM-1 by T cell-expressed TCR and LFA-1, respectively (51). For MHC class II, the MIIC tubules can orientate selectively towards the immunological synapse in mouse DCs and depends on TCR and ICAMs (48–50). Finally, the F-actin cytoskeleton of the DCs is also polarized towards the immunological synapse in an antigen-specific manner, and this polarization is required for complete T cell activation (52,53). Thus, it is increasingly clear that reverse MHC-I and MHC-II signaling trigger several polarized trafficking cascades: extension of endosomal networks (recycling endosomes and MHC-II), rearrangements of the F-actin cytoskeleton, and recruitment of the MTOC and the accompanying Golgi network.

Altogether the MTOC and Golgi polarization leads to T cell activation. During this interaction the combination of cytokines sensed by the T cell define its differentiation into various types of effector or repressor T cells (54). For instance, IL-12 mediates the differentiation of $CD4^+$ helper T cells to Th1 cells for clearance of (mainly) viruses and other intracellular pathogens, whereas TNF-α and IL-6 promote a differentiation to Th2 and Th17 phenotypes, which are more important for extracellular pathogens and humoral responses (54). Additionally, many other cytokines, including IL-4, IL-10 and TGF-β, are involved in differential T cell activation (54). However, lymph nodes are very crowded organs that contain millions of T cells and only a very small fraction, as low as one in a million, of these T cells carries a TCR recognizing a particular epitope (55). Although the DC usually interacts with several T cells at the same time in the so called rosette structure (7,56), only one at the time gets activated. This arises the question how does the DC prevent bystander activation? Our hypothesis is that the DC delivers a variety of signals directly to one antigen-specific T cell at the time via the MTOC and Golgi trafficking, which might prevent bystander activation of the other T cells in the rosette. Thus, our mechanism explains how most cytokines can be locally released at the immunological synapse, explaining how DCs can direct specific T cell differentiation in crowded cellular environments.

Materials and methods

Animals

C57BL/6 wild-type (WT) and OT-I mice were purchased from Charles River and hosted at the Central Animal Laboratory (CDL) of the Radboud University Medical Center. The mice were kept in top-filter cages and received a standard diet. At the time of an experiment, the mice were sacrificed by cervical dislocation at an age of 8-12 weeks old. All experiments in this project were approved by an animal ethical committee [2015-0019TIL-067] with regard to the care and use of animals.

Murine cell culture

For the generation of BMDCs, murine femurs and tibias were taken from female WT mice. From the femurs and tibias, bone marrow derived stem cells were obtained and cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS, 758093, Greiner bio-one), 1% antibiotic-antimycotic (15240-062, Gibco), 1% ultraglutamine (BE17-605E/U1, Lonza Bioscience), 50 μM β-mercaptoethanol (60-24-2, Sigma-Aldrich) and 200 ng/ml human Flt3L (130-096-479, Miltenyi Biotec) for eight days at 37°C in an incubator with 10% CO_2. After eight days, floating cells were collected and a CD45R (B220) depletion (microbead isolation kit (130-049-501), Miltenyi Biotec) was performed to remove plasmacytoid DCs from the cell population. For the isolation of naive $CD8^+$

T lymphocytes, spleen and lymph nodes were isolated from female OT-I mice. The organs were homogenized and digested for 30 minutes at 37ºC by using a mixture of collagenase and DNAse, at 1 mg/ml and 130 µg/ml, respectively. $CD8^+$ T cells were obtained after LD column magnetic antibody cell sorting (MACS, 130-042-901, Miltenyi Biotec) with anti-CD8 antibody beads from Miltenyi Biotec (130-104-075). The beads were used according to the company's protocol.

Human cell culture

Human cells were obtained from a buffy coat from healthy individuals received as anonymous coded specimens from Sanquin Bloodbank Nijmegen and were handled according to practice and legal guidelines. The research with human blood samples at the Department of Tumor Immunology complies with all institutional and national ethics regulations and has been approved by the ethics committee of Sanquin. All blood donors were informed of the research and have granted their consent. Density grading media Ficoll facilitated the isolation of leukocytes. After a series of centrifugation and washing steps with cold PBS with 1 mM EDTA (6381-92-6, Sigma-Aldrich) and 1% human serum (47-196, Sigma-Aldrich), the peripheral blood mononuclear cells (PBMCs) were obtained. To generate moDCs, the PBMCs were plated and left to attach for 1 hour at 37 ºC, whereafter the floating cells (lymphocytes) were removed. The moDCs were cultured for six days in complete medium with 300 U/ml interleukin 4 (IL-4, 130-093-924, Miltenyi Biotec) and 450 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, 130-093-867, Miltenyi Biotec) at 37 ºC. After three days, new complete medium with 900 U/ml IL-4 and 1350 U/ml GM-CSF was added.

BMDC-T cell synapse formation

Fresh day 8 Flt3L BMDCs ($B220^-$) were activated in a non-adherent well plate (7007, Corning) for four hours at 37ºC and 5% CO_2 with LPS (L4391-1MG, Sigma-Aldrich) and CpG ODN1826 (tlrl-1826-1, InvivoGen) both at a concentration of 1 µg/ml. The cells either received no addition of protein/peptide, OVA albumin (0.5 mg/ml, 321001, Lionex GmbH), OVA in combination with IgG against OVA (20 µg/ml OVA + 500 µg/ml Goat polyclonal IgG against chicken ovalbumin, 0855303, MP Biomedicals) or SIINFEKL (500 ng/ml, $OVA_{257-264}$, AS-60193-5, Tebu-Bio). OVA and OVA-immunocomplexes were added for the full activation time, while SIINFEKL was added for the last 30 minutes of activation. After activation, OT-I T cells obtained from mouse spleen and lymph nodes and labelled with CFSE (C34554, Invitrogen) were added 2:1 to the BMDCs and placed on PLL-coated coverslips. The DCs and T cells were incubated for 1 hour at 37°C and 5% CO_2. After this time the cells were fixed for 20 minutes with 4% PFA.

BMDC-bead synapse formation

Fresh day 8 Flt3L BMDCs ($B220^-$) were activated in a non-adherent well plate for four hours at 37°C and 5% CO_2 with TLR4 agonist LPS and TLR9 agonist CpG-B ODN1826 both at a concentration of 1 µg/ml. During activation, 6 µm streptavidine beads were coated with different biotinylated antibodies (3.75 µg of antibody per 6 million beads, see table 1.) and incubated for 30 minutes in PBA (PBS + 1% BSA and 0.05% sodium azide) at room temperature (RT). After activation, the BMDCs were mixed with beads on poly-L-lysine (PLL, 25988-63-0, Sigma-Aldrich) coated coverslips in a 1:1 ratio and were incubated for 30 minutes at 37°C and 5% CO_2. After this time the cells were fixed for 20 minutes with 4% paraformaldehyde (PFA). Bead coating was tested with flow cytometry (Appendix 1).

Table 1 The antibodies and protein domains used in the BMDC-bead experiments.

Target	Antibody	Catalog number	Company
MHC-I	Rat anti-mouse H-2 monoclonal antibody (biotinylated)	MBS2533680	MyBioSource
Isotype MHC-I	Biotin Rat IgG2a, kappa isotype control clone R35-95	553928	BD Biosciences
MHC-II	Biotin rat-anti mouse I-A/I-E clone 2G9	553622	Sigma Aldrich
ICAM-I	ICAM-I/CD54 antibody (1A29) biotinylated	NBP2-22541B	Novus Biologicals
LFA-I	Monoclonal anti-human IgG1-biotin antibody + recombinant mouse ICAM-I-Fc chimera (carrier free)	B6775+553006	Biolegend
CD80/86	Monoclonal anti-human IgG1-biotin antibody + recombinant mouse CD28-Fc chimera (carrier free)	B6775+755606	Biolegend

MoDC-bead synapse formation

Day 6 moDCs were activated in a non-adherent well plate for four hours at 37°C and 5% CO_2 with LPS and TLR7/8 agonist R848 at a concentration of 1 µg/ml and 2.5 mg/ml respectively. After activation, the moDCs were mixed with beads coated with either an antibody against MHC-I (13-9983-82, ThermoFisher) or its corresponding isotype control (13-4724-85, ThermoFisher) on PLL-coated coverslips in a 1:1 ratio and were incubated for 30 minutes at 37°C and 5% CO_2. After this time the cells were fixed for 20 minutes with 4% PFA. The coating of the beads was analyzed with flow cytometry (Appendix 1).

Immunofluorescence staining of MTOC

After fixation with PFA for 20 minutes at RT, excess PFA was quenched with a solution containing 100 mM glycine and 100 mM NH_4Cl in PBS for 20 minutes at RT. Human DCs were blocked-permeabilized with 2.5% BSA + 1% donkey serum (017-000-121-,

Jackson) + 0.15% Triton X-100 in PBS, mouse DCs were blocked-permeabilized with 2.5% BSA + 1% donkey serum + 0.1% Triton X-100 in PBS. Synapses from murine BMDCs were stained with antibodies against α-tubulin (1:500, rat IgG, NB100-1639, Novus Biological),γ-tubulin (1:500, rabbit IgG, T5192, Sigma-Aldrich) and the cis-Golgi marker GM-130 (1:100, mouse IgG, 610822, BD Pharmigen) for one hour at RT. After washing, the synapses were stained with fluorescently labelled secondary antibodies: donkey-anti-rat alexa488 (1:400, A21208, Thermo-Fisher Scientific) donkey-anti-rabbit alexa568 (1:400, A11036, Thermo-Fisher Scientific) and donkey-anti-mouse alexa647 (1:400, A10037, Thermo-Fisher Scientific). After incubation with the secondary antibody, the cells were washed with PBS and mounted with a glycerol-containing mounting medium (68 g glycerol, 100 mM NaPi pH 7.4 + trollox + DAPI).

Human cells were stained for α-tubulin (1:500, rat IgG, NB100-1639, Novus Biological), γ-tubulin (1:500, rabbit IgG, T5192, Sigma-Aldrich) and the trans-Golgi marker TGN46 (1:1000, sheep IgG, AHP500GT, BioRad) for one hour at RT. After washing, the synapses were stained with fluorescently labelled secondary antibodies purchased from Thermo-Fisher Scientific: donkey-anti-rat alexa488 (A21208), donkey-anti-rabbit alexa568 (A11036) and donkey-anti-sheep alexa647 (1:400, A21448). Lastly, the cells were washed with PBS and mounted with a glycerol-containing mounting medium (68 g glycerol, 100 mM NaPi pH 7.4 + trollox + DAPI).

Electron Microscopy

Synapses of mouse BMDCs and T cells were seeded and cryo-fixed on aluminum discs by plunging rapidly in liquid propane. Cells were dehydrated and fixed using the rapid freeze substitution method (K.L. McDonald, R.I. Webb Freeze substitution in 3 hours or less) in the presence of 1% OsO4 and 0.5% Uranyl acetate in acetone containing 5% water. Samples were embedded in epon and 100 nm sections were collected on formvar coated and carbon evaporated grids. Sections were post contrasted with uranyl cetate and lead citrate and examined in a CM12 transmission electron microscope (Philips) operating at 100kV. Synapses were imaged on an SP8 confocal microscope with a 63x 1.2 NA water immersion objective (Leica).

In-plate MHC-I activation for Western Blot

A six well plate was coated with 15 µg/µl BSA (10735108001, Roche), 15 µg/µl of MHC-I antibody (BE0077, BioXCell) and 15 µg/µl of isotype control antibody (400202, BioLegend) in PBS overnight at 4°C. On the day of the experiment, fresh day 8 mouse Flt3L BMDCs ($B220^-$) were activated in a non-adherent 6 well plate (3471, Corning) coated with 10% BSA for four hours at 37°C and 5% CO_2 with LPS and CpG-B ODN1826 at a concentration of 1 µg/ml. 45 minutes before the end of activation, the wells were blocked with blocking buffer (2% BSA in PBS) at 37°C and 5% CO_2. After activation, the

BMDCs were added to the wells and the plate was centrifuged to ensure synchronized contact between the antibody and the cells. The plate was incubated for 30 minutes at 37 ºC and 5% CO_2. The cells were collected and lysed with lysis buffer (1% Nonident P-40, 100 mM Tris pH 7.5, 150 mM NaCl supplemented with phosphatase (04-906837-001, Roche) and protease inhibitors (05-892-791-001, Roche)) and incubated for 20 minutes on ice. The lysates were collected and wells were washed 3 times with lysis buffer. Sample buffer without β-mercaptoethanol was added to each well and the plate was incubated for 10 minutes in a water bath at 60ºC.

The lysates and complex fractions were run on a 10% (for p-Tyr) and 15%-SDS gel. The samples were transferred to polyvinylidene difluoride (PVDF) membranes. After the transfer, the membranes were washed with demineralized water and were blocked with Odyssey blocking buffer (927-40000, LI-COR: Tris-buffered saline (TBS) 10mM Tris, pH 8.0, 150 mM NaCl, 5% BSA with 0.2% Tween-20) for 1h at RT. After blocking, the membranes were incubated with antibodies against phospho-tyrosine (SC-508, Santa Cruz), GAPDH (2118, Cell Signaling) or MHC-I (HLA-A, ABIN3023100, Antibodies Online) overnight at 4ºC. Additionally, the membranes were washed with TBS-Tween [*concentration) and incubated with fluorescently labelled secondary antibodies goat-anti-mouse IRdye800 (926-32210, LI-COR), donkey-anti-rabbit IRdye 680 (925-68073, LI-COR) and goat-anti-rabbit IRdye 800 (925-32211, LI-COR) for one hour at RT. The membranes were washed with TBS-Tween and scanned using the Odyssey CLx, a LiCor infrared blot imager. Analysis of the blot was done with ImageJ.

Statistical analysis

The plots for polarization index show the average ± standard error of the mean (SEM). For the statistical analysis of the polarization index; D'Agostino & Pearson omnibus normality test was performed followed by one-way analysis of variance (ANOVA) with Bonferroni's Multiple Comparison Test or Kruskal-Wallis followed by Dunnet. Significance is displayed in the graphs and a value of $p < 0.05$ was considered statistically significant.

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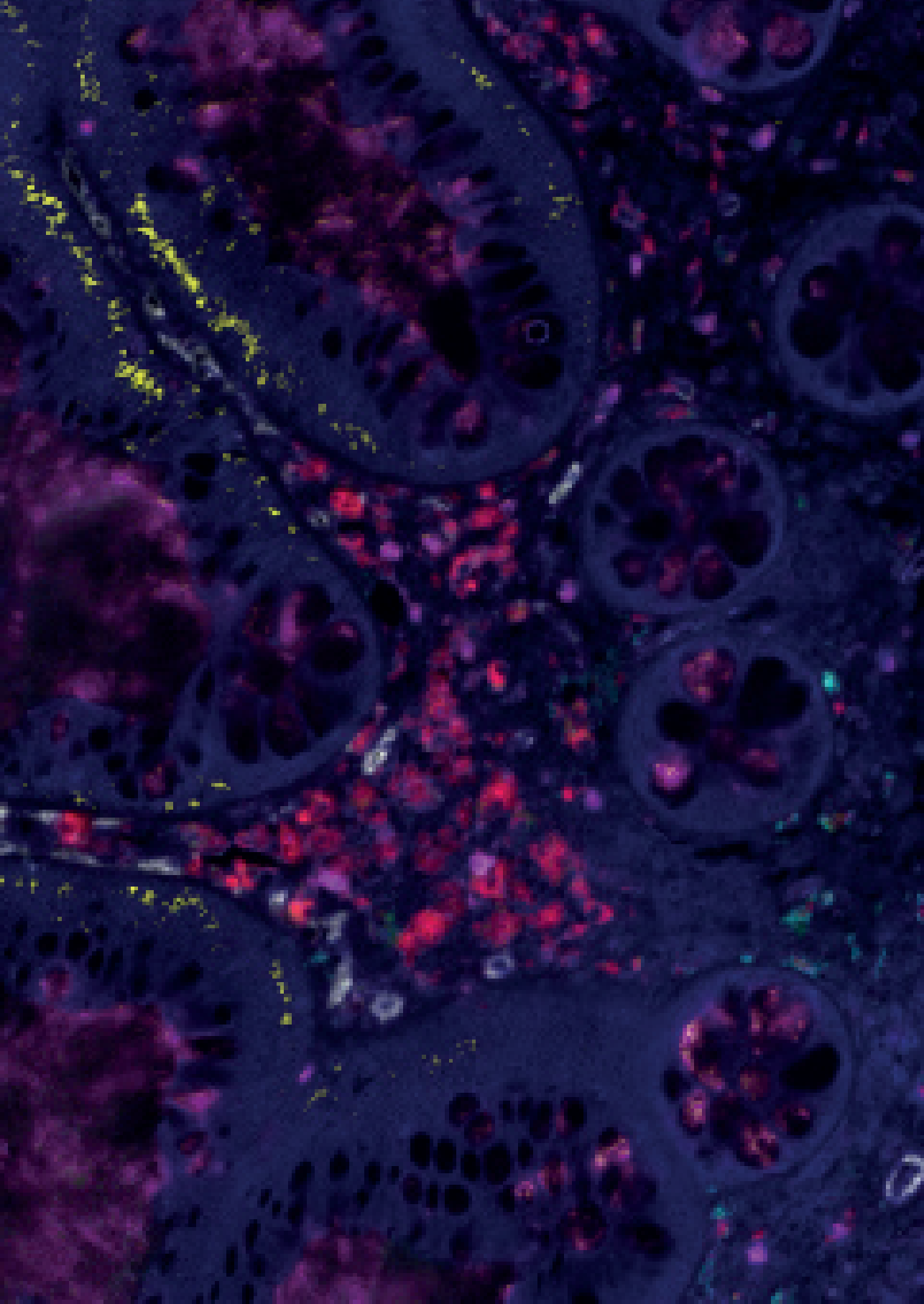

Chapter 8

General discussion

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Manuscript in preparation

Abstract

Catestatin (CST: $hCgA_{352-372}$), a bioactive cleavage product of the neuroendocrine pro-hormone Chromogranin A (CgA), is associated with pathological conditions hallmarked by chronic inflammation, including autoimmune diseases, cardiac diseases and metabolic disorders. CST is an anti-inflammatory and anti-adrenergic peptide, and the concept is emerging that CST primarily acts by suppressing the inflammatory actions of macrophages. However, recent findings show that CST not only acts on macrophages, but that macrophages themselves are main producers of CST, possibly producing even more CST than neuroendocrine cells. In this opinion article, we discuss new data supporting the exciting possibility that macrophages are the main source of CST for suppression of inflammation and downregulation of catecholamine release by neuroendocrine cells.

Introduction

Chromogranin A (CgA) is produced and secreted by neuroendocrine and enteroendocrine cells, such as chromaffin cells and pancreatic beta-cells (1–5). Proteolytic conversion of CgA, either before or after its release, gives rise to six known bioactive cleavage products: pancreastatin (PST: $hCgA_{250-301}$), WE-14 ($hCgA_{324-337}$), vasostatin (hCgA1-76), catestatin (CST: $hCgA_{352-372}$), chromofungin ($hCgA_{47-66}$) and serpinin ($hCgA_{411-436}$) (5,6). The exact regulation of CgA cleavage is not clear, but various proteases have been shown to be capable of producing CST, including cathepsin-L (7), thrombin (8), furin (9), pro-hormone convertases 1 and 2 (9), kallikrein (10) and plasmin (11,12). Although the receptor and downstream signaling pathway for CST remain unknown, the physiological effects of the 21-amino acid peptide CST are well established. CST is pleiotropic and functions as a neuroregulatory, anti-bacterial as well as chemotactic peptide, regulates insulin sensitivity and has anti-inflammatory effects in various inflammatory models, including diabetes, inflammatory bowel disease (IBD), rheumatoid arthritis (RA) and cardiac disease (13–26). It is believed that by producing CST, neuroendocrine cells regulate their own activation by negative-feedback inhibition, and suppress inflammation in a paracrine fashion (6,27).

However, in this opinion article, we argue that CST mainly signals in the reverse direction: from macrophages to neuroendocrine cells. First, we will discuss the role of CST in suppressing catecholamine release and macrophage inflammation. Second, we will discuss new evidence suggesting that macrophages are major producers of CST. Third, we will discuss the contribution of this macrophage-produced CST to the regulation of the neuroendocrine and immune systems. Finally, we touch upon the potential clinical implications of CST in chronic inflammatory diseases.

The role of CST in suppression of neurotransmitter release

CST was first described as a neuropeptide (27,28) present in for example the retina and nerves of the eye (29). Additionally, it was found that neuroendocrine cells and adrenergic neurons, which both release catecholamines, co-released these together with CST (27,28,30). Supporting this co-release, plasma CST levels are accepted as a biomarker for sympathetic nervous system activity (31), where high CST levels correlate with increased neurohumoral burden (31,32). Neurons and neuroendocrine cells do not only produce CST, but CST also affects their activity. For example, CST stimulation decreased sympathetic nerve activity, heart rate, sympathetic barosensitivity and neuronal response to hypoxia in rats stimulated in the pressor area of the medulla (33) as well as improved baroreflex sensitivity and heart variability in mice (34,35). Moreover, mice with selective deletion of the region of the *Chga* gene coding for CST, so called CST-KO mice, are hypertensive and have elevated levels of

norepinephrine and epinephrine in plasma and the adrenal gland, which are fully reversible by intraperitoneal injection of CST (25). This suppression of catecolamine release by CST might well be a direct effect on the neurons and/or neuroendocrine cells, because CST was found to suppress catecholamine release in the *in vitro* cultured neuronendocrine cell line PC12 (30) as well as *in vivo* in mice (36). Based on these findings, CST is considered to be an autocrine neuromodulatory factor that enables neuroendocrine cells to self-suppress their catecholamine release in an activity-based fashion (6,27,30).

The role of CST in suppression of inflammation

CST also suppresses inflammation and is thereby involved in different diseases and disorders hallmarked by chronic inflammation (Fig. 1). For instance, plasma CST levels are elevated in patients suffering from type 2 diabetes (37), hypertension (38), heart failure (31,39–41), sleep apnea (42) and acute lung infection (32). Elevated levels of CST have also been measured in blood and stool of IBD patients (13,18,22), and these levels seem to correlate with disease severity (14). In animals, high CST levels in the saliva correlate with cortisol, indicating physiological stress (43–45). CST-KO mice display elevated levels of pro-inflammatory cytokines in the circulation, adrenal gland, colon and heart, which could all be reversed upon intraperitoneal injection of CST (23,25). Experiments with fluorescently-labelled dextran showed that CST-KO mice also have an impaired gut barrier function, a hallmark of intestinal inflammation, which was reversed by CST administration (13). Finally, *in vitro* cultured murine bone marrow-derived macrophages were more skewed to an anti-inflammatory phenotype in the presence of CST (25). Based on these findings, it is believed that the co-release of CST together with catecholamines acts to dampen inflammation upon elevated neurohumoral activity (6,27,30–32).

CST controls inflammation via macrophages

The anti-inflammatory effects of CST primarily occur by suppression of monocyte infiltration and macrophage-driven inflammation. CST-KO mice have increased macrophage infiltration in the adrenal gland, heart, and colon (13,23,25) (Fig. 1). Depletion of the macrophages in CST-KO mice, using chlodronate liposomes, reversed the hypertensive phenotype and normalized the blood pressure (25). Intraperitoneal injection of CST leads to a reduction of monocyte and macrophage infiltration in the colon (colitis model), liver (diet-induced obesity model), heart (hypertension model) and atheromatous plaques (atherosclerosis model) (13,14,20,21,23–25) (Fig. 1). Intravital imaging and *in vitro* assays revealed that CST directly affects monocyte chemotaxis (15–17,23). Key evidence showing that macrophages are the main cells regulated by CST, comes from bone-marrow transfer experiments from CST-KO mice to irradiated wild-type mice. Despite the wild-type mice having completely

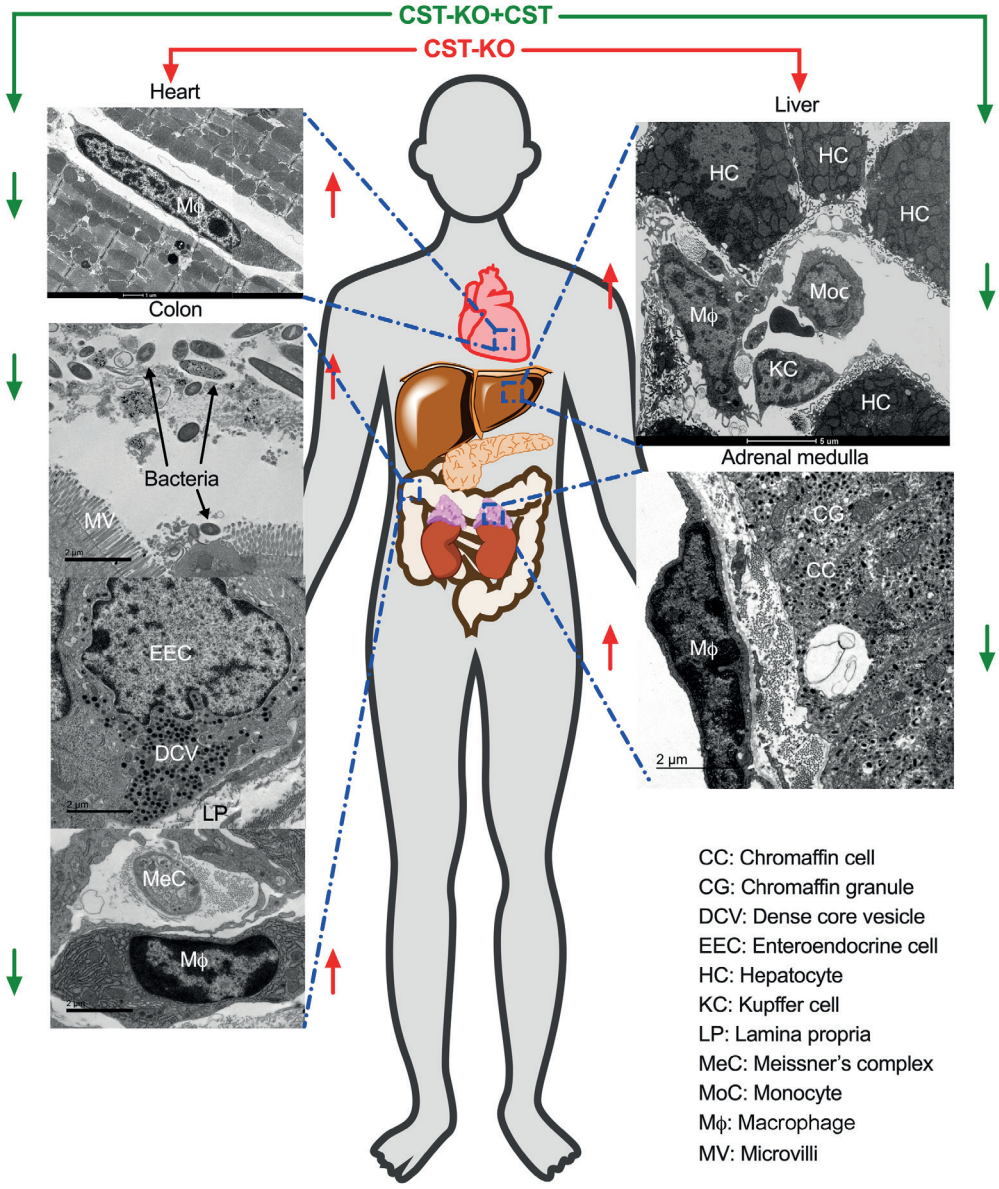

Fig. 1 The effects of catestatin on several tissues. Tissues that are affected upon CST knockout (red) and CST treatment (green). **Heart:** Macrophage (MΦ) infiltration in absence of CST (red). **Colon:** Affected microvilli and (MV) mucus production, microbiome composition and epithelial barrier function and increased macrophage infiltration upon absence of CST (red). **Liver:** Hepatocytes (HC) with more macrophage and monocyte (MoC) infiltration in absence of CST (red). **Adrenal medulla:** Macrophage infiltration upon CST knockout (red).

autologous CST production from the neuroendocrine cells, this transfer resulted in elevated levels of proinflammatory cytokines in the heart (the only organ assessed) and induced a hypertensive phenotype (22,25). In contrast, bone marrow transfer in the reverse direction (i.e., from wild-type to CST-KO mice) resulted in the opposite phenotypes (22,25), proving that the metabolic and inflammatory effects of CST are largely due to its suppression of macrophage-driven inflammation.

Macrophages could be the main source of CST

The bone-marrow transfer experiments (22,25) also revealed another important fact: macrophages (and/or other bone-marrow derived cells) are major producers of CST themselves. This was shown by the following two observations: 1) the inflammatory and hypertensive phenotypes were fully reversible upon bone marrow transfer from WT to CST-KO mice, showing that CST produced by bone marrow-derived cells is sufficient for these phenotypes, and 2) the levels of CST in cirulation almost normalized following transfer (25). Moreover, since bone-marrow transfer from CST-KO mice to WT mice could induce the inflammatory and hypertensive phenotypes, this suggests that CST produced by the macrophages is essential for the physiological function of CST (25). The production of CST by murine peritoneal macrophages was confirmed by Western blot (25).

Thus, it seems that macrophages are both main cells affected by and major producers of CST, supporting the conclusion that CST might be a new autocrine signaling factor supressing macrophage activation. This extends the current idea that CST is mainly produced by neurons and neuroendocrine cells when it is secreted together with catecholamines, and this suppresses macrophage-driven inflammation in an autocrine/ paracrine fashion (28,30).

Macrophage-produced CST suppresses neurotransmitter release

If macrophages are the main source of CST for dampening inflammation, this raises the questions what the contribution of CST produced by neuroendocrine cells is. Does the CST produced by neuroendocrine cells result in self-suppression of catecholamine release and dampen macrophage inflammation, as previously envisioned for catecholamine secretion induced by nicotine or PACAP (pituitary adenylate-cyclase-activating polypeptide) (6,27,30,36)? Or alternatively, does CST produced by macrophages regulate the activity of neurons and neuroendocrine cells? Recent evidence also supports the latter possibility, since bone-marrow transfer from WT mice to CST-KO mice resulted in normalization of norepinephrine and epinephrine levels in the plasma and adrenal gland, whereas bone marrow transfer in the reverse direction resulted in elevated levels of these catecholamines (25). Macrophages are well known to communicate with neuroendocrine cells using cytokines (46–48), and CST thus seems to be a new factor mediating this communication (Fig.2).

Especially for the intestine, it seems likely that macrophage-produced CST regulates both the enteric nervous system and enteroendocrine system (49–52). In the gut, CST affects the bacteria population, intestinal barrier function, macrophage infiltration and inflammation (13,14,19–22) and possibly intestinal mobility (unpublished data). Many of these processes are regulated by the enteric neuroendocrine system, such as intestinal motility, blood flow, mucosal growth and the immune system (46,47). Thereby, it seems likely that CST produced by macrophages could signal to this system resulting in both reduced inflammation and gastrointestinal activity (Fig.2).

Concluding remarks

Based on the above discussion, we propose that macrophages are one of the main source of CST which controls inflammation in an autocrine fashion and neuronal/neuroendocrine activity in a paracrine fashion (Fig.2). However, a limitation of the current studies in mice is that complete knockout mice for CST were used. This model does not allow to discern between CST produced by neurons, neuroendocrine cells and macrophages. A conditional CST knockout in these different cell types using a Cre/loxp model (53) could help to discern their contribution in CST production.

Moreover, conditional knockout of CST would help to distinguish between contributions of locally vs. systemically produced CST and delineate the roles of CST in different organs. Since levels of CST in plasma, feces and saliva might be used as biomarkers in several inflammatory diseases (13,14,18,31,32,37–41,43–45), it will be important to understand these contributions. The specific roles of CST in different organs might also be assessed by reducing the local levels of CST in WT mice with injections of neutralizing antibodies. Alternatively, local CST concentrations could be raised by injection of (coated) vesicles containing CST or RNA coding for CST targeted with antibodies to distinct organs (54).

Direct administration of CST could help patients with over activated macrophages in various inflammatory diseases. However, peptides are rapidly degraded in the circulation and in general have a low half-life. The use of so called retro-inverso-CST (RI-CST) might overcome this limitation (55). This peptide contains D-amino acids instead of L and has a reversed sequence, but could still rescue hypertension in CgA-KO mice similarly as seen for normal CST (55). In contrast to normal CST, RI-CST is not efficiently broken down by proteases in the digestive tract and thereby likely has an extended half-life and might even be administered orally. Additionally, a pharmacophore-based screen identified TKO-10-18 as a potential drug that can mimic CST actions (56). Thus, the administration of RI-CST or TKO-10-18 might be promising therapies for diseases with inflammatory and neuroendocrine dysfunctions, including metabolic disorders and autoimmune diseases such as diabetes and RA.

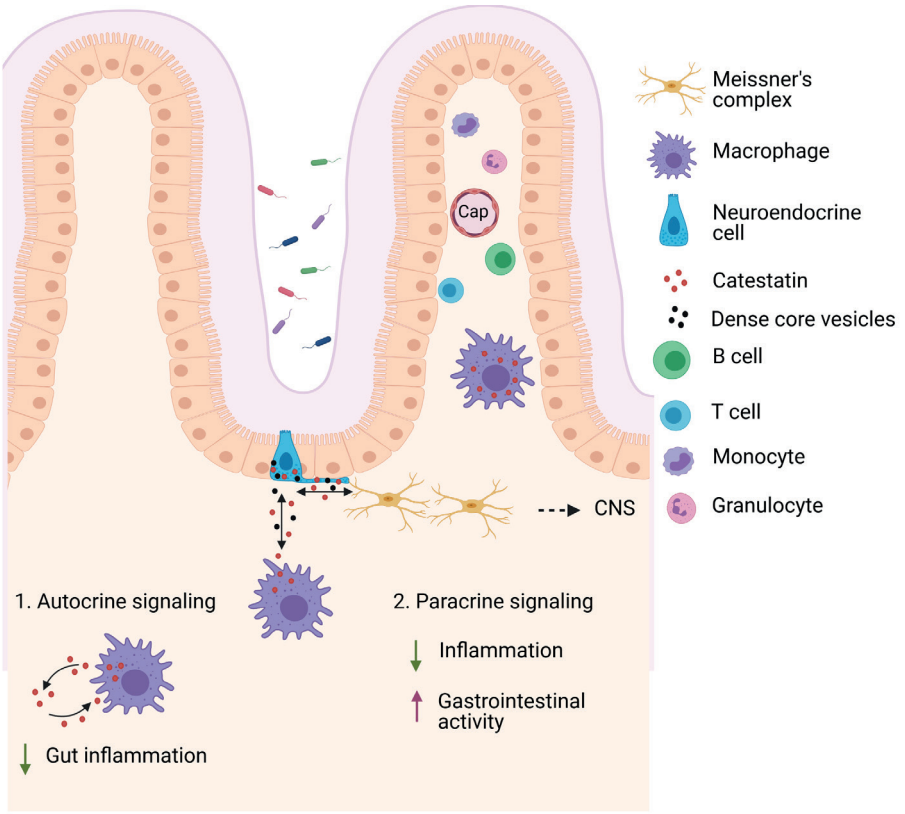

Fig. 2 CST mediates communication between macrophages and the enteric neuroendocrine system. CST mediates the communication between macrophages and neuroendocrine cells resulting in reduced gastrointestinal activity and reduced inflammation. These reactions can occur via 1) autocrine signaling, where CST is produced by the macrophages and affects the macrophages directly, and 2) paracrine signaling from macrophages to neuroendocrine cells, resulting in suppression of central nervous activity (CNS) via the Meissner's complex (created with BioRender.com).

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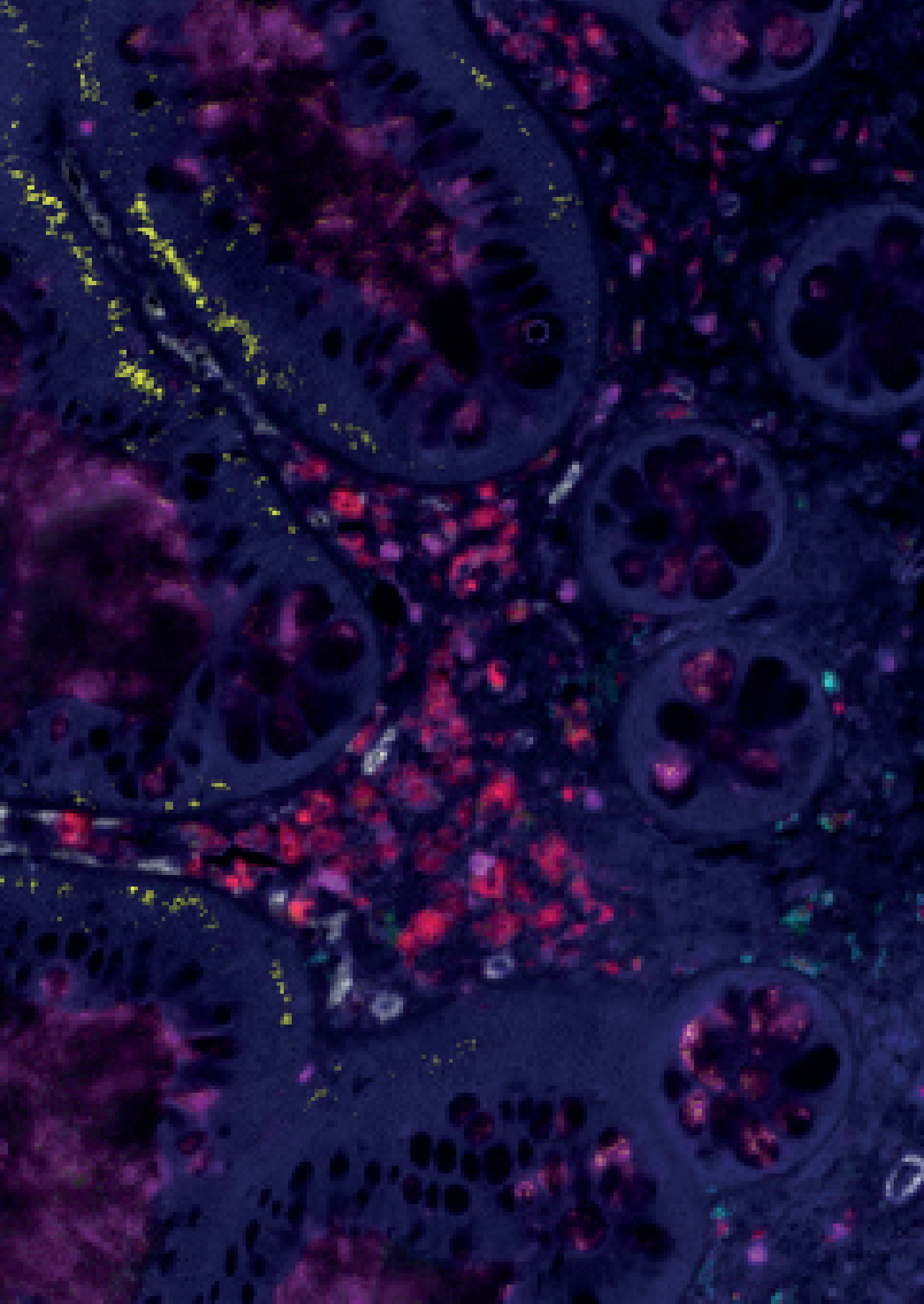

Appendices

Nederlandse samenvatting

Dit proefschrift gaat over de effecten van catestatin op ontsteking in de darm en de rol van MHC-I in de verdediging tegen tumoren en virussen.

De rol van catestatin tijdens ontsteking

Het hormoon chromogranine A wordt geproduceerd door verschillende gespecialiseerde cellen in het lichaam, zoals in de eilandjes van Langerhans in de alvleesklier. Na productie kan dit hormoon in de volgende actieve stukjes worden geknipt (Fig. 1); pancreastatin (PST: hCgA250-301), WE-14 (hCgA324-337), vasostatin (hCgA1-76), catestatin (CST: hCgA352-372), chromofungin (hCgA47-66) en serpinin (hCgA411-436), deze stukjes noemen we peptiden.

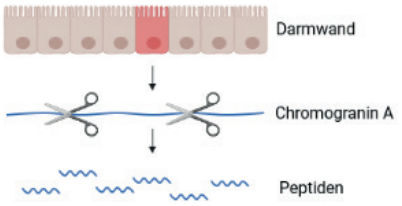

Fig. 1. Productie van chromogranine A door cellen in de darmwand, dat na productie in zes actieve stukjes geknipt kan worden.

Op dit moment is er veel belangstelling voor de peptide catestatin, omdat deze een belangrijke rol lijkt te spelen in het onderdrukken van ontsteking in het lichaam. Zo zijn er zelfs afwijkende hoeveelheden van catestatin in het bloed gemeten van patiënten met een hartafwijking, stofwisselingsziekte of chronische ontsteking zoals in de ziekte van Crohn, collitis ulcerosa, diabetes en reumatoïde artritis.

De effecten van catestatin op de darmwand

Patienten met ziektes als IBD (ziekte van Crohn en collitis ulcerosa), prikkelbaredarmsyndroom en coeliakie hebben patiënten vaak last van ontsteking en een 'lekkende' darmwand. De darmwand, bestaande uit een laag cellen met daarop een slijmlaag, beschermt in een normale situatie het lichaam tegen indringers zoals bacteriën en virussen. Maar wanneer de beschermende laag beschadigd wordt, kunnen bacteriën door de darmwand heen. Dit leidt tot een zogeheten ontstekingsreactie waarbij witte bloedcellen naar de darm komen om de bacteriën uit te schakelen en ze op te ruimen. Bij een chronische darmontsteking kan de ontsteking niet opgelost worden, waardoor patiënten last blijven houden van hun darmen en vaak levenslang medicatie nodig hebben.

Catestatin beïnvloedt de barrièrefunctie en slijmlaag van de darmwand

In **hoofdstuk 2** bekijken we de effecten van catestatin en pancreastatin op de regulatie van de darmwand. We komen tot de volgende vier bevindingen:

1) Na onderzoek op bloed van patiënten met de ziekte van Crohn en collitis ulcerosa blijkt dat de hoeveelheden chromogranine A en catestatin in deze patiënten hoger zijn dan bij de gezonde controlegroep

2) De darm van muizen die geen catestatin kunnen maken, bevat minder soorten bacteriën. Interessant genoeg wordt ditzelfde patroon in de bacteriën ook gezien voor IBD-patiënten

3) We kijken naar de zogeheten tight junctions (alsof de cellen elkaars handen vasthouden), dit zijn de connecties tussen de eenlagige darmcelwand. Deze blijken langer te zijn in muizen die geen catestatin kunnen produceren. De tight junctions lijken bovendien op de connecties tussen darmwandcellen van IBD-patiënten

4) Het 'lekken' van de darm is erger in muizen die geen catestatin kunnen maken. Door middel van het toedienen van pancrestatin of catestatin aan muizen zonder chromogranine A zien we dat het lekken van de darm zelfs tegenovergesteld gereguleerd wordt. Het toedienen van pancreastatin leidt tot meer 'lekken', terwijl het toedienen van catestatin juist tot minder darmlekkage leidt.

We concluderen hieruit dat chromogranine A en zijn actieve peptiden belangrijk zijn voor de functie van de darmwand. Catestatin speelt een belangrijke rol in dit proces door zijn effecten op de tight junctions, de aanwezige bacteriën en het transport over de darmwand.

Naast de barrièrefunctie van de darmwand kijken we naar de regulatie van de slijmlaag in **hoofdstuk 3**. Hier laten we zien dat catestatin, geproduceerd door immuuncellen uit het beenmerg, de slijmlaag reguleert. Daarnaast hebben IBD-patiënten afwijkende hoeveelheden catestatin in hun ontlasting. Hieruit leiden we af dat de verdwijnende slijmlaag in ontstoken delen van de darm gereguleerd wordt door catestatin.

Het effect van catestatin op de migratie van immuuncelmigratie

In **hoofdstuk 4** kijken we naar het effect van catestatin op de zogeheten immuuncelmigratie (verplaatsing) tijdens ontsteking. Hier maaken we gebruik van verschillende technieken waarbij we bijvoorbeeld de omgeving konden controleren of de cellen *live* volgen in de muis na stimulatie met catestatin. Hiermee laten we

zien dat catestatin normaal gesproken immuuncellen aantrekt, maar de migratie van immuuncellen naar een ontstoken omgeving blokkeert. Deze bevindingen zijn in lijn met het idee dat catestatin helpt tijdens een ontsteking.

Het werkingsmechanisme van catestatin

Gedurende dit onderzoek kwamen we erachter dat catestatin niet alleen wordt geproduceerd door gespecialiseerde cellen in de celwand, maar ook door immuuncellen met een oorsprong in het beenmerg. In de discussie van dit proefschrift, **hoofdstuk 8,** speculeren we daarom over: 1) De bijdrage van deze verschillende cellen op de anti-inflammatoire werking van catestatin; 2) De interactie tussen het zenuwstelsel en het immuunsysteem via catestatin; 3) Mogelijke behandelingsopties voor de toekomst met oraal toedienbare catestatin; 4) Opties voor vervolgonderzoek om het precieze werkingsmechanisme te achterhalen.

De interactie tussen immuuncellen ter verdedigen tegen tumoren en virusinfecties.

In het tweede deel van dit proefschrift komt de interactie tussen speciale immuuncellen, dendritische cellen en macrofagen, om de verdediging tegen tumoren en virale infecties te activeren aanbod. In **hoofdstuk 5** beschrijven we al het huidige onderzoek naar de rol van verscheidene macrofagen uit het bloed en uit weefsel (o.a. lever, vet en lymfeklier) in het doorgeven van informatie aan de T-cellen. De T-cellen kunnen met deze informatie de gevaarlijke cellen, zoals een tumorcel, uitschakelen. Het was al bekent dat macrofagen lokaal helpen bij het oplossen van een ontsteking door bv. bacteriën op te eten, maar dat ze net als dendritische cellen ook informatie kunnen overdragen aan T-cellen is nieuw. Dit maakt macrofagen interessant om te gebruiken voor de behandeling van virusinfecties of kanker.

De functie en interactie van MHC-I in de verdediging tegen tumoren en virusinfectie

Hierna bespreken we in **hoofdstuk 6** de rol van de receptor MHC-I in de verdediging tegen kanker en virussen. MHC-I is aanwezig op immuuncellen zoals macrofagen, dendritische cellen en T-cellen, maar ook op andere soorten cellen zoals gladde spiercellen, endotheel en tumorcellen. Activatie van MHC-I zet signalering aan die kan leiden tot het doodgaan van de cel of doodmaken van andere cellen, tot celdeling of celmigratie naar een andere plek. In **hoofdstuk 7**, bestuderen we de effecten van MHC-I op de interactie tussen de dendritische cel en de T-cel. Deze immuuncel interactie maakten we zichtbaar met behulp van een elektronenmicroscoop die zeer kleine structuren in de cel zichtbaar maakt. Vervolgens laten we met behulp van metalen bolletjes beplakt met MHC-I zien, dat MHC-I ervoor zorgt dat structuren in

de dendritische cel verplaatsen en leiden tot cytokinen productie. Deze cytokinen activeren vervolgens de T-cel, die op zijn beurt de tumor of geïnfecteerde cel kan uitschakelen.

Conclusies

Catestatin beinvloedt de bacteriën, darmwand en migratie van cellen tijdens ontsteking. Ondanks dat we niet precies weten hoe catestatin voor deze effecten zorgt, kunnen we wel verschillen meten in de catestatinwaarden in het bloed en ontlasting van patiënten. Hierdoor is catestatin interessant als indicator om de ziekteprogressie te meten. Daarnaast zorgde het toedienen van catestatin voor het terugdraaien van de ontsteking. Dit maakt catestatin interessant voor de ontwikkeling van toekomstige behandelingen voor patienten met een chronische ontsteking.

Het activeren van T-cellen is nodig voor het uitschakelen van gevaarlijke cellen. Om de huidige therapieën te verbeteren zouden naast dendritische cellen ook macrofagen gebruikt kunnen worden voor T-cel activatie. Daarnaast speelt MHC-I een belangrijke rol in de immuuncelinteractie die leidt tot de activatie van T-cellen. Het begrijpen van dit mechanisme helpt om de werking van het immuunsysteem verder te ontrafelen.

Layman summary (published in Fair journal)

The hormone Chromogranin A balances gut wall function

Introduction

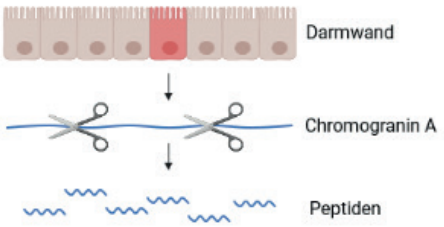

Fig. 1. Chromogranin A production and cutting resulting in the active peptides.

In diseases related to chronic inflammation such as; inflammatory bowel disease (IBD), irritable bowel syndrome, and celiac disease, patients suffer from a 'leaky' gut wall. Normally, the gut wall consists of a one-layer cell-wall with a mucus layer on top that protects you against invaders in the gut, such as pathogenic bacteria. If this protection is damaged, the bacteria are no longer kept outside and can now enter our body. This triggers an inflammatory reaction where white blood cells move towards the gut to kill the invaders and repair the damaged or 'leaky' gut wall. In the case of chronic gut inflammation this damage cannot be repaired, which results in ongoing bacterial invasion and damage of the gut. As a result, these patients suffer from complications due to ongoing gut inflammation and need lifelong treatment.

In search for a treatment, we focussed on understanding how the gut wall is kept intact at steady-state (also called homeostasis). Here, we took a closer look at the locally produced hormone chromogranin A (Fig. 1). After production by specialized cells in the gut wall, chromogranin A is cut into six small active pieces; pancreastatin, WE-14, vasostatin, catestatin, chromofungin and serpinin. All these active pieces, which are called peptides, play a role in regulating health and disease.

Interestingly, the two peptides catestatin and pancreastatin seem to have opposing effects; where catestatin plays a role in suppressing inflammation and pancreastatin seems to stimulate inflammation. However, we do not currently know much about their function in controlling gut homeostasis. Therefore, we investigated the role of the active peptides catestatin and pancreastatin on the 'leaky' gut wall.

Findings

To be able to study the specific role of the complete hormone chromogranin A and its peptide catestatin in a physiological setting, we made so-called *chromogranin A* knockout (full hormone-KO) and *catestatin* knockout (peptide-KO) mice, meaning that

we removed the genes that codes for the hormone or its peptide. The full hormone-KO mouse could no longer make chromogranin A, while the peptide-KO mouse could still make chromogranin A, but it could no longer cut it into the peptide catestatin. Additionally, we could feed normal or knockout mice a fluid containing only the full hormone or peptide to study its effect as a kind of medication. By using both mouse types and peptides as supplementation we could create specific situations necessary to study the role of chromogranin A and catestatin on the gut wall function.

First, we looked at the so called 'tight junctions', which are structures in the gut cells that keep them tightly together in one line (like the cells are holding hands). By using an ultrastructural microscope, that is able to enlarge even the most tiny structures inside the cells, we could see that the tight junctions in the CST-KO mouse were longer and looked similar as in inflammatory bowel disease patients.

Secondly, we studied how 'leaky' the gut-wall was by measuring how much sugar made it to the other side of the gut-wall (the sugar was labelled with a green dye to be able to see it). Here, detection of green on the other side means 'leaky', because in a healthy situation the gut-wall is intact and we would not expect to detect green sugar on the other side. We learnt that the peptide catestatin is important for maintaining the gut barrier since the mice without catestatin showed a very 'leaky' gut. By treating the full hormone-KO mouse with either catestatin or pancreastatin, we could even see that the leakiness of the gut is oppositely regulated by these two peptides; meaning that in presence of catestatin the gut-wall seems intact, while after addition of pancreastatin the wall seems 'leaky'.

Thirdly, we identified the bacteria types present in the gut. In the peptide-KO mouse we found a higher amount of *Firmicutes* and a lower amount of *Bacteroidetes* than in the normal mouse. Normally, *Firmicutes* are necessary for energy uptake from food, whereas *Bacteroidetes* produce acids to keep a good environment in the gut for food uptake. However, these bacteria populations need to be balanced to be able to take up food and keep the gut in a steady-state. Interestingly, the same shift in bacteria groups we found in the peptide-KO mouse is also observed in inflammatory bowel disease patients. This shows us that the presence of catestatin is necessary to maintain a healthy balance in the gut bacteria that is present.

Finally, we measured that chromogranin A hormone and catestatin peptide levels in the blood of inflammatory bowel disease patients were elevated when compared to healthy people.

Conclusions

Altogether, the hormone chromogranin A and its active peptides play an important role in maintaining the gut wall function. Especially the peptide catestatin plays a role in making the gut wall less 'leaky' by affecting the length of the tight junction, the presence of bacteria, and the transport over the gut-wall barrier. Additionally, catestatin seems to exert its effect by opposing another peptide pancreastatin. Although we now know a great deal more about how these molecules affect gut homeostasis, more research is still necessary to define the exact working mechanism.

Our research is important to understand how gut homeostasis is regulated in health and disease. Additionally, blood catestatin levels might be used as a biomarker in the future to indicate the presence of inflammation in inflammatory bowel disease patients. Finally, catestatin and pancreastatin might be interesting in the future to develop treatments for inflammatory bowel disease and other gut-related diseases.

Reviewed by Jonas N. Søndergaard (editor at the Fair Journal).

Research data management

All primary research data as presented in this thesis were archived according to the FAIR (Findable, Accessible, Interoperable and Reusable) principles. All data are stored digitally on a local server of the Tumor Immunology department of the Radboud University Medical Center (RadboudUMC) and experiments are described in the online lab book system Labguru. All data and documents stored on the local server are accessible to the supervisors and associated scientific staff. All raw data of research performed in the RadboudUMC as used in Chapter 2 is also available through the open-source repository Zenodo. The raw data of Chapter 3, 4 and 7 will be available on Zenodo upon publication.

The research with human blood samples at the Department of Tumor Immunology complies with all institutional and national ethics regulations and has been approved by the ethics committee of Sanquin blood bank. All blood donors were informed of the research and have granted their consent. For chapter 2 and 3, informed consent was obtained and the study was conducted in accordance with the Institutional Board Review of the University Medical Center Utrecht. All samples were collected in compliance with the Declaration of Helsinki.

All procedures relating to the WT and KO mice were approved by the Institutional Animal Care and Use Committee (UCSD) and all procedures, housing and handling of the animals were in accordance with National Institutes of Health animal care guidelines. Experiments in chapter 4 using male and female C57BL/6J (Taconic, Denmark) and Cx_{3cr1}^{GFP} mice were approved by the Regional Animal Ethics committee in Uppsala, Sweden.

Curriculum Vitae Elke M. Muntjewerff

Elke Marjolein Muntjewerff was born on 9th of June 1992 in Nijmegen, The Netherlands. After completing her secondary education in 2010, she started her bachelor studies on Biomedical Sciences at Utrecht University including a minor to become a certified Biology teacher. Afterwards, she started the Master programme 'Infection and Immunity' at the Utrecht University.

As part of her master she performed a nine months internship on the role of enterovirus and norovirus proteinases during infections at the Virology department in Utrecht under supervision of Dr. Martijn Langerijs and Prof. Frank van Kuppenveld. Followed by a six months internship at the Institute for Allergy and Immunology in San Diego, US. Here she studied the movement of regulatory T cells in the pancreas in diabetes type 1 under supervision of Dr. Gustaf Christoffersson and Prof. Mathias von Herrath.

After obtaining her master's degree Elke worked as a researcher in the group of Dr. Daniel Sidler and Prof. Dr. Uyen Hynh-Do at the department of Nephrology in the Inselspital University Hospital Bern, Switserland. Here she worked on a research line focussed on a screen to predict the degree of liver involvement in female patients with autosomal dominant polycystic kidney disease.

In 2018 she started her PhD project at the department of Immunology at the Radboud Institute for Molecular Life Sciences in Nijmegen. Under supervision of Prof. Geert van den Bogaart en Prof. Carl Figdor she performed the research as presented in this thesis. During her PhD she obtained several travel grants allowing her to attend conferences and visit her co-supervisor Dr. Sushil Mahata at the University of California San Diego in the US. Additionally, the EMBO grant gave her the opportunity to perform research at the Uppsala University, Sweden. Here she worked under supervision of her other PhD co-supervisor Dr. Gustaf Christoffersson.

After finishing her PhD thesis, Elke continued her projects as post-doctoral researcher at the University of Groningen under the supervision of Prof. Geert van den Bogaart.

Elke is currently starting a new position as post-doctoral researcher at the cell biology department at the Uppsala University, Sweden under the supervision of Dr. Gustaf Christoffersson.

Published articles

Muntjewerff EM, Tang K, Lutter L, Christoffersson G, Nicolasen MJT, Gao H, Katkar GD, Das S, ter Beest M, Ying W, et al. Chromogranin A regulates gut permeability via the antagonistic actions of its proteolytic peptides. *Acta Physiol* (2021) **232**:e13655. doi:10.1111/apha.13655

Muntjewerff EM, Meesters LD, Bogaart G Van Den, Revelo NH. Reverse Signaling by MHC-I Molecules in Immune and Non-Immune Cell Types. *Front Immunol* (2020) **11**:1–20. doi:10.3389/fimmu.2020.605958

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Baranov MV, Bianchi F, Schirmacher A, van Aart MAC, Maassen S, **Muntjewerff EM**, Dingjan I, ter Beest M, Verdoes M, Keyser SGL, et al. The Phosphoinositide Kinase PIKfyve Promotes Cathepsin-S-Mediated Major Histocompatibility Complex Class II Antigen Presentation. *iScience* (2019) **11**: doi:10.1016/j.isci.2018.12.015

Muntjewerff EM, Dunkel G, Nicolasen MJT, Mahata SK, Van Den Bogaart G. Catestatin as a Target for Treatment of Inflammatory Diseases. *Front Immunl* (2018) **9**:2199. doi:10.3389/fimmu.2018.02199

Available on bioarchive

Muntjewerff EM, Lutter L, Tang K, Lindert MK, Fransen J, Oldenburg B, Mahata SK, van den Bogaart G. Catestatin regulates the colonic mucus layer in inflammatory bowel disease. (2021) doi:doi.org/10.1101/2021.02.09.430377

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Articles in preparation

Muntjewerff EM, Christoffersson G, Mahata SK, Van Den Bogaart G. Autocrine regulation of macrophage inflammation by catestatin.

Muntjewerff EM, Lutter L, Maassen S, Warner H, Nicolasen MJT, Stevens M, ter Beest M, Mahata SK, Van Den Bogaart G. Catestatin promotes mitochondrial elongation and metabolism in human macrophages.

Muntjewerff EM, Nicolasen MJT, Bogaart G Van Den, Revelo NH. MHC class I reverse signalling triggers polarized release of IL-12 via MTOC polarization.

PHD PORTFOLIO

Name PhD candidate:	**PhD period:**
E.M. Muntjewerff	01-04-2017 – 01-05-2021
Department:	**Promotor(s):**
Tumor Immunology	Prof. C. Figdor &
Graduate School:	Prof. G. van den bogaart
Radboud Institute for Molecular Life Science	**Co-promotor(s):**
	Dr. G. Christoffersson
	Dr. S.K. Mahata

	Year(s)	ECTS
TRAINING ACTIVITIES		
a) Courses & Workshops		
• Introduction day Radboudumc	2017	0.5
• Graduate School specific introductory course (RIMLS)	2017	1.0
• Opfris cursus statistiek in excel (RU PhD course)	2017	1.5
• Expert Flow Cytometry Training, 2 days basic course (in Nijmegen)	2017	1.0
• ENII Immunology Summer school (incl. poster presentation)	2018	1.75
• Cytometry workshop, RAI Amsterdam	2018	1.75
• Personal Brand Key (Twinq)	2018	4.0
• Scientific Integrity course (RIMLS)	2019	1.0
• Loopbaanmanagement voor Promovendi	2019	1.5
• Within Sight of my PhD (RIMLS)	2019	1.0
b) Seminars & lectures		
• RPI lunch seminar personal experiences on grant writing (RIMLS)	2017	0.1
• Seminar Prof. Dr. Hans Clevers (Genmab, Utrecht)	2017	0.1
• MMD Masterclasses/Mini-symposium (TIL, RIMLS)	2017	0.2
• Seminar Sophie Acton, UCL London	2017	0.1
• Webinar advanced flow cytometry experiments (Excyte)	2018	0.1
• Webinar 4-18 color compensation (Excyte)	2018	0.1
• Webinar Advanced data analysis (Excyte)	2018	0.1
• Radboud Research Rounds	2018	0.1
• Semniar Anastasiya Schirmacher	2018	0.1
• Wiebke Herzog (Uppsala, Zweden)	2018	0.1
• Seminar Susanne Gabrielsson (Uppsala, Zweden)	2018	0.1
• Seminar Robbert Spaapen (RIMLS)	2018	0.1
• Radboud Research Rounds	2019	0.1
• Seminar Daan Vorselen (RIMLS)	2019	0.1

c) Symposia & congresses		
• PhD retreat	2017	0.5
• Radboud New Frontiers; microbiome (RIMLS)	2017	0.5
• Science for life conference Utrecht (incl. oral presentation)	2017	0.5
• ICI 2018, Chemical Immunology, Amsterdam	2017	0.25
• PhD retreat (incl. poster) (RIMLS)	2018	0.75
• DC2018 (incl. poster), Aachen, Germany	2018	1.5
• ECI2018, Amsterdam (incl. poster)	2018	1.0
• Uppstart 2018, Uppsala, Zweden	2018	0.5
• Science day Groningen (incl. oral presentation)	2018	0.5
• Immunology 2019 (incl. poster) San Diego, US	2019	1.75
• Innate Immunity (incl. poster) Tubingen, Germany	2019	1.25
• Dutch Immunology meeting (incl. Talk), Breukelen	2019	0.75
• NVVI Noordwijk 2019 (incl. poster), Noordwijk	2019	0.75
• ISSCB (invited talk), Chennai, India	2020	1.75
• Science day Groningen (incl. oral presentation)	2020	0.5
• NVVI Noordwijk 2020 (incl. talk), online	2020	0.75
• NVVI Lunteren meeting 2020, online	2021	0.5
• Innate Immunity eKeystone (incl. Sci-talk), online	2021	1.75
d) Other		
• Journal clubs Tumor Immunology	2017	0.5
• Journal clubs Tumor Immunology (incl. paper presentation)	2018	1.0
• Journal clubs Tumor Immunology (incl. paper presentation)	2019	1.0
• Journal clubs Tumor Immunology (incl. paper presentation)	2021	1.0
TEACHING ACTIVITIES		
e) Lecturing		
• Practicum minor students BMS (MIN02, DC transfection)	2017	0.5
• Practicum master students (BMS36 IF, edit your own gene)	2017	0.5
• Research proposal writing (MIN04)	2019-2020	0.7
• Research proposal writing (MIN04)	2020-2021	0.7
f) Supervision of internships		
• Supervision master student (Sjors Maassen)	2017-2018	2.5
• Supervision bachelor honours student (Mara Nicolasen)	2018	0.7
• Supervision master thesis (Gina Dunkel)	2018	1
• Supervision master student (Lieke der Haer)	2018-2019	2.5
• Co-supervision master student (Mara Nicolasen)	2019	1.5
• Supervision master thesis (Luca Meesters)	2019	0.75
• Supervision bachelor honours student (Milou Stevens)	2020	1
TOTAL		**48.1**

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